Role of Leukocytes During Hematogenous Metastasis

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Content

Abbreviations

CIS	Carcinoma In Situ
DMEM	Dulbecco's Modified Eagle's Medium
EC	Endothelial Cells
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid
EMT	Epithelial Mesenchymal Transition
FCS	Fetal calf serum
FGF	Fibroblast Growth Factor
GalNAc	N-acetylgalactosamine
GFP	Green Fluorescent Protein
GlcNAc	N-acetylglucosamine
HEV	High Endothelial Venules
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
IBD	Inflammatory Bowel Disease
ICAM	Intercellular Adhesion Molecule
IL	Interleukin
L-/P-/E-sel	L-/P-/E-selectin
L-/P-/E-sel-/-	L-/P-/E-selectin deficient
MCP	Monocyte Chemotactic Protein
MMP	Matrix Metalloproteinase
NFκB	Nuclear Factor κB
NK cells	Natural Killer cells
NSAIDs	Nonsteroidal anti-Inflammatory Drugs
PMA	Phorbol 12-Myristate 13-Acetate
PNAd	Peripheral Node Addressins
PSGL-1	P-selectin Glycoprotein Ligand-1
RANKL	Receptor Activator of NFκB Ligand
RPMI	Roswell Park Memorial Institute medium
SCF	Stem Cell Factor
sLe^x	sialyl-Lewis x
TAA	Tumor-associated antigens
TAM	Tumor-associated Macrophages
TC	Tumor Cells
TCM	Tumor-conditioned medium
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

Summary (English)

Metastasis is the main cause of cancer-related death. To metastasize and form new foci, tumor cells have to enter the bloodstream, escape from the immune system and extravasate through the vascular endothelium. Tumor cell extravasation is supported by the formation of tumor emboli which are aggregates of tumor cells with platelets and leukocytes. These aggregates protect tumor cells from clearance by the immune system, thereby enhancing metastasis. Platelet adherence on tumor cells is mediated by P-selectin and platelets contribute considerably to metastasis. Although leukocytes have been observed in interaction with tumor cells, their role remained to be elucidated. Attenuation of metastasis was observed in L-selectin deficient mice, implicating leukocytes as potential facilitators. In this project, we aimed to define the nature and function of leukocytes in the tumor cell emboli as possible mediators of metastasis based on the observation that leukocytes were associated with tumor cells in lung tissues. We established a transmigration assay of tumor cells in presence of leukocytes *in vitro* and showed that depending on L-selectin expression, both granulocytes and monocytes were contributing to tumor cell transmigration. This enhanced migration was associated with an increased permeability of the vascular endothelium. The function of granulocytes and monocytes was then investigated *in vivo*. Depletion of monocytes led to a strong attenuation of metastasis, implicating monocytes as an important contributor of metastasis. Taken together these data provide evidence for an involvement of leukocytes in metastasis and indicate that L-selectin mediates this process.

Zusammenfassung (Deutsch)

Die meisten Krebspatienten sterben aufgrund der Metastasierung von Tumorzellen. Um zu metastasieren und neue Metastasen zu bilden, müssen Tumorzellen in die Blutbahn eintreten, die Immunabwehr umgehen und durch die Blutgefäßwand wandern. Die Diapedese von Tumorzellen ist durch die Bildung von sogenannten Tumorzellemboli begünstigt, welche aus Aggregaten von Plättchen und Leukozyten um die Tumorzellen bestehen. Diese Aggregate helfen den Tumozellen dem angeborenen Immunsystem zu entgehen und einen Metastase zu generieren. Plättchen interagieren mit Tumorzellen über P-Selektin und tragen zur Metastasierung bei. Obwohl Leukozyten mit Tumorzellen interagieren, bleibt Ihre Rolle unklar. Die Reduktion der Metastasierung in L-Selektin defizienten Mäusen deutet auf Leukozyten als potentielle Vermittler hin. Das Ziel dieses Projektes war es, die Natur und die Funktion der Leukozyten in Tumorzellemboli zu bestimmen und ihre Beteiligung an der Metastasierung nachzuweisen. Leukozyten sind mit Tumorzellen in Lungengewebe assoziiert. Wir untersuchten die *in vitro* Funktion der Leukozyten und konnten nachweisen, dass in Abhängigkeit von der L-Selektin Expression sowohl Granulozyten, als auch Monozyten zur Tumorzelltransmigration beitragen. Die verstärkte Migration ist mit einer erhöhten Blutgefässdurchlässigkeit korreliert. Die Granulozyten- und Monozytenfunktionen wurden dann auch *in vivo* untersucht. Eine Depletion der Monozyten führt zu einer verminderten Metastasierung, was diesen einen massgeblichen Beitrag zur Metastasenbildung bescheinigt. Diese Resultate zeigen dass durch L-Selektin und demnach durch Leukozyten Metastasierung begünstigt wird.

Section I: INTRODUCTION

1 Cancer, an inflammatory disease

1.1 Epithelial cancer / carcinoma

The majority of human tumors arise from epithelial tissues. These tumors, called carcinomas are responsible for more than 80 % of cancer-related deaths in the Western World (Weinberg, 2007). The other 20 % are due to sarcomas, myelomas, leukemias and lymphomas. Carcinomas are classified into two major categories, reflecting the two major biological functions associated with epithelia. Tumors that arise from epithelial cells forming protective cell layers are called squamous cell carcinomas (e.g., cancer of the skin or esophagus). Carcinomas originating from epithelial cells of glandular tissue (like mucous glands from the lungs and the stomach) are called adenocarcinomas.
Migration of cancer cells from the original tumor site through the blood and lymph vessel to produce cancers in other tissues is called metastasis and is the leading cause of death due to cancer. An earlier detection of cancer could reduce the death by metastasis.

Hanahan and Weinberg proposed six physiological changes that most if not all cancers acquire during their development. These changes are 1) the insensitivity to anti-growth signals, 2) the self-sufficiency of growth signals, 3) the evasion from apoptosis 4) the limitless potential to replicate, 5) the sustained angiogenesis, and 6) tissue invasion and metastasis (Hanahan and Weinberg, 2000). Tumor progression is driven by a sequence of alterations affecting cell proliferation, adhesion, survival, invasion and migration, all processes being associated with the acquisition of a malignant phenotype. The aim of this project was to study mechanisms leading to the initiation of metastasis.

1.2 Chronic inflammation associated with cancer

In 1863, Rudolf Virchow suggested a connection between cancer and chronic inflammation after the observation of leukocytes in neoplastic tissues (Balkwill and Mantovani, 2001). Tumors are considered as `wounds' that do not heal (Dvorak, 1986). Thus, inflammation and accompanying inflammatory cells and cytokines are commonly involved in tumor promotion and progression.

Chronic inflammation can lead to human carcinomas (**Table 1)**. For example, aetiologic agents, like chronic airway irritation and tobacco smoke are promoters of lung cancer. Infectious agents, like hepatitis B and C viruses and accompanying inflammation of the liver are associated with hepatocellular carcinomas, whereas infections with *Helicobacter pylori* are associated with most gastric cancers. Underlying infections and inflammation are linked to 15-20 % of all cancer deaths (Kuperwasser et al., 2004).

Pathologic condition	**Associated neoplasm**	**Aetiologic agent**
bronchitis	lung carcinoma	silica, smoking (nitrosamines, peroxides)
inflammatory bowel disease Crohn's disease chronic ulcerative colitis	colorectal carcinoma	
chronic pancreatitis hereditary pancreatitis	pancreatic carcinoma	alcoholism, mutation in trypsinogen gene
skin inflammation	melanoma	ultraviolet light
cancer associated with infectious agents		
gastritis, ulcers	*gastric adenocarcinoma*	*Helicobacter pylori*
hepatitis	*hepatocellular carcinoma*	*hepatitis B and / or C virus*
mononucleosis	*B-cell non-Hodgkin's lymphoma* *Burkitts lymphoma*	*Epstein-Barr virus*
AIDS	*non-Hodgkin's lymphoma* *squamous cell carcinoma* *Kaposi's sarcoma*	*human immunodeficiency virus* *human herpes virus type 8*

Table 1: *Chronic inflammatory conditions associated with neoplasms.* Figure adapted from (Coussens and Werb, 2002).

One good example of inflammation-associated cancer is the inflammatory bowel disease (IBD) –associated cancer. IBD makes reference to two diseases due to an altered host

response to normal intestinal bacterial flora: ulcerative colitis and colonic Crohn's disease which are both associated with risk for colorectal cancer (Rhodes and Campbell, 2002). There is a strong correlation between the duration and extent of inflammation of the mucosa and the cancer risk. The gut is the largest lymphoid organ in the body and in germ-free animals the colon inflammatory cells were greatly reduced indicating that a 'normal' colon can be seen as being in a perpetual state of inflammation **(Figure 1)**.

As a consequence of this observation, inhibition of inflammation could then reduce the incidence of sporadic cancer. It was indeed shown that daily ingestion of aspirin reduces by half the risk of sporadic colon cancer (Dubois, 2000). Later, clinical studies report that the inhibition of chronic inflammation by nonsteroidal anti-inflammatory drugs (NSAIDs), particularly inhibitors of inducible cyclo-oxygenase COX-2, were promising as anticancer drugs (Pham-Nguyen et al., 1999). COX-2 has indeed a favorable effect to cancer development by inhibiting apoptosis and promoting angiogenesis (Rhodes and Campbell, 2002).

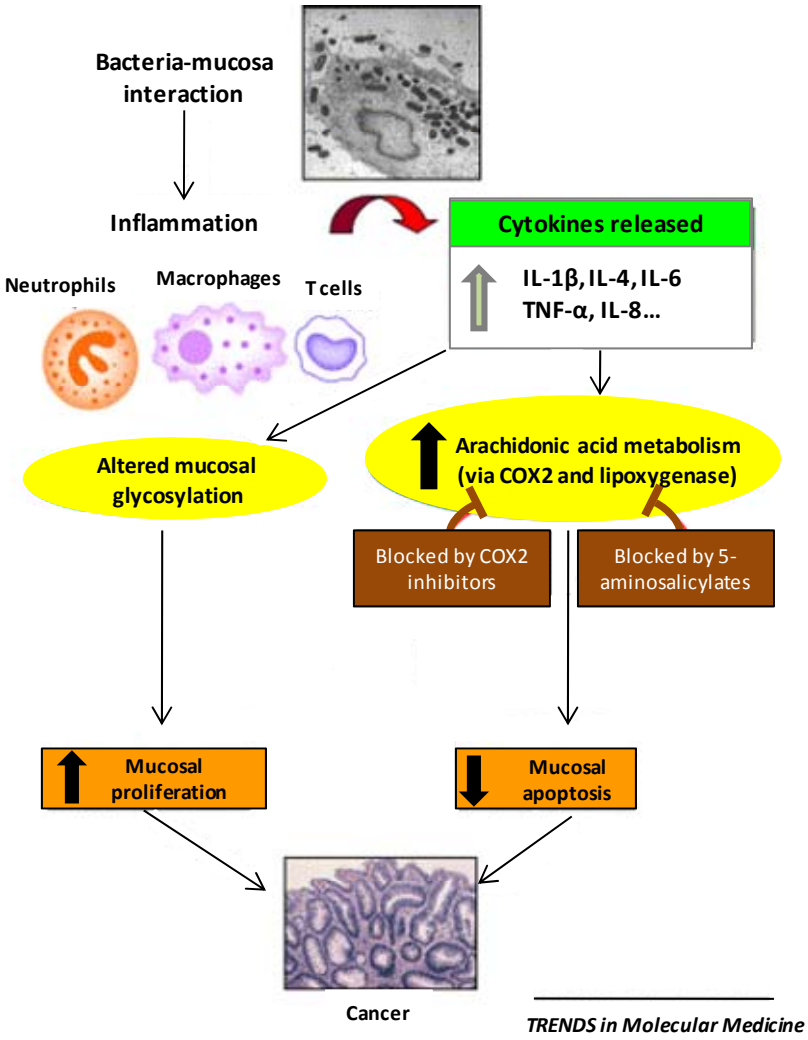

Figure 1: *Mechanisms for colon cancer development as a consequence of inflammation.* Colon cancer is associated with underlying inflammation like IBDs. Cytokines released by this inflammatory environment lead to an increase in mucosal proliferation and to a reduction in mucosal apoptosis thereby increasing the chance of cancer development. Figure adapted from (Rhodes and Campbell, 2002).

Cytokines mediate underlying inflammations and attract immune cells on the site of primary tumor. Tumor cells interact with these inflammatory cells to support their growth and development. The resulting dialogue leads to an enhanced tumor progression.

1.3 Cytokines in tumor progression

During chronic inflammation, tissue damage or chronic infection, both tumor cells and tumor-associated leukocytes and platelets produce inflammatory cytokines (like tumor necrosis factor TNF, IL-1 and -6) and chemokines (like IL-8). These cytokines and chemokines may facilitate cancer progression as illustrated by the case of the cytokine TNF. It selectively destroys blood vessels at high doses, whereas when it is chronically produced, it may contribute to tissue remodeling and tumor growth **(Figure 2)**. For example, mice depleted for TNF were resistant to skin carcinogenesis (Moore et al., 1999) and in a model of ovarian cancer, TNF-α, constitutively expressed on the malignant epithelium, promoted cancer growth and spread (Kulbe et al., 2007). In clinic, TNF-α antagonists give encouraging results in patients with advanced myeloma (Singhal et al., 1999).

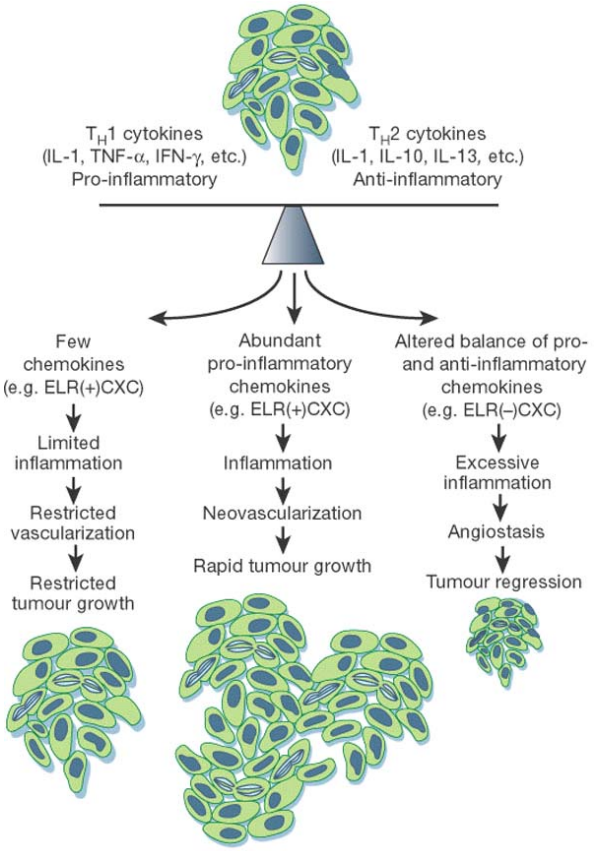

Figure 2: *The knowledge of the cytokine balance is crucial to determine cancer progression.* Cytokines can have a pro- or an anti-inflammatory effect, leading respectively to two opposite effects: rapid tumor growth or tumor regression. Figure reprinted from (Coussens and Werb, 2002).

Chemokines are chemotactic cytokines, that can be expressed by carcinomas and which are able to attract leukocytes to inflammation sites (Balkwill, 2004; Kakinuma and Hwang, 2006). Immune cells, endothelial cells and tumor cells express chemokine receptors and are able to respond to chemokine gradients, like neutrophils, that migrate in the presence of CXCL8 (IL-8). Chemokines contribute also to tumor cell growth, as illustrated by IL-8, that acts as autocrine growth factor for melanoma, liver, pancreatic and colon cancer cells (Schadendorf et al., 1995). Many chemokine receptors are involved in metastasis and CXCR4 is the most common chemokine receptor overexpressed in human cancers **(Table 2)**. It is implicated in lung metastasis; indeed, mice injection of CXCR4-expressing cancer cells leads to metastasis to lungs whereas injection of tumor cells treated with a neutralizing antibody against CXCR4 leads to an attenuation of metastasis (Muller et al., 2001). CXCR4 can be activated by its ligand CXCL12 which has been shown to increase metastatic properties, growth and survival of tumor cells (Scotton et al., 2002).

Chemokine receptor	Site of metastasis	Cancer cell type	Involvement
CXCR4	lung bone lymph node	23 different cancers*	migration growth/ survival angiogenesis
CCR4	skin	Sezary cells (leukemic CTCL)	migration
CCR7	lymph node	melanoma gastric, esophageal CLL lung	migration
CCR9	small intestine	melanoma, prostate	migration
CCR10	skin	melanoma CTCL	migration growth/survival

Table 2: *Chemokine Receptors in Cancer Metastasis.* Chemokine receptors overexpressed in human cancers are implicated in tumor progression and metastasis. Table reprinted from (Kakinuma and Hwang, 2006).

* Breast cancer, ovarian cancer, glioma, pancreatic cancer, prostate cancer, acute myeloid leukemia, B-chronic lymphocytic leukemia, B-lineage acute lymphocytic leukemia, non-Hodgkin's lymphoma, intraocular lymphoma, follicular center lymphoma, chronic myelogenous leukemia, multiple myeloma, thyroid cancer, **colorectal cancer,** squamous cell cancer, neuroblastoma, renal cancer, astrocytoma, rhabdomyosarcoma, small-cell lung cancer, melanoma, and cervical cancer. Abbreviations: CTCL: Cutaneous T cell lymphoma; CLL: chronic lymphocytic leukemia.

1.4 Interactions of primary tumors with their microenvironment

The developing tumors have a complex relationship with the cells from their microenvironment (de Visser et al., 2006). In the early growth of tumors, cancer cells form a neoplastic lesion that is embedded in the epithelium, separated from the surrounding tissue and contained within the boundary of a basement membrane (Hanahan and Weinberg, 2000). This is called the carcinoma *in situ* (CIS). The basement membrane, the immune cells, the capillaries, the fibroblasts, and extracellular matrix (ECM) surrounding the cancer cells constitute the tumor stroma (Ronnov-Jessen et al., 1996). This stroma is comparable to that observed in wound healing and is called reactive stroma. There is evidence that the reactive stroma and CIS can communicate with each other through the basement membrane barrier (Ronnov-Jessen et al., 1996). It is not yet fully understood how CIS becomes an invasive carcinoma by degrading the basement membrane (Kalluri, 2003). Reactive stroma is associated with an increased number of fibroblasts, enhanced capillary density, and type-I-collagen and fibrin deposition and gives oncogenic signals that facilitate tumorigenesis (Dolberg et al., 1985). Vascular endothelial growth factor (VEGF) plays a role in the emergence of the reactive stroma (Brown et al., 1999).VEGF is released by cancer cells, fibroblasts and inflammatory cells leading to the recruitment of blood vessels by tumor cells, a process called angiogenesis. It has been shown in a mouse model, that inflammatory cells are then recruited and release MMP-9 that cleaves ECM components and liberates latent inactive VEGF from its sequestered state (Nozawa et al., 2006). VEGF acts on endothelial cells causing them to proliferate and invade hypoxic tumor tissues expressing hypoxia-inducible factor 1 (HIF-1) in order to supply them with blood vessels. The resulting enhancement in microvascular permeability of the tumor-associated capillaries and venules and extravasation of plasma proteins such as fibrin attract an influx of fibroblasts, inflammatory cells and endothelial cells (Dvorak et al., 1991). During cancer progression, the tumor cells invade the reactive stroma and come into direct contact with fibroblasts, inflammatory cells and newly formed capillaries (Dvorak, 1986). Carcinoma-associated fibroblasts (CAFs) are playing a role in the initiation and progression of cancer, as well as in

metastasis (Kalluri and Zeisberg, 2006). The **figure 3** shows the microenvironment of tumor cells and potential new therapeutics directed towards deprivation of tumor cells stromal support.

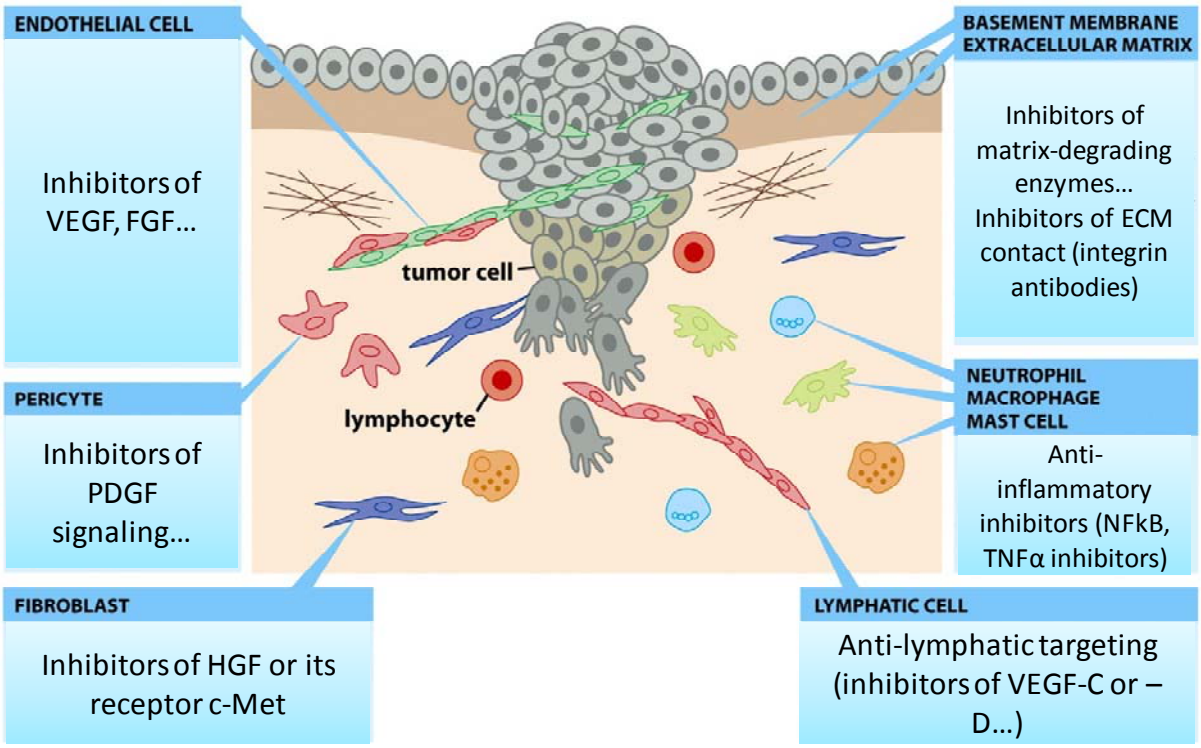

Figure 3: *Heterotypic interactions as targets for therapeutic intervention*. Cancer cells are dependent on the nearby environment for a variety of cell physiologic supports. This dependence on heterotypic interactions has inspired a new type of cancer therapy. Instead of focusing on intracellular defects within cancer cells, this new type of therapy is directed towards depriving tumor cells of their essential stromal support. This scheme indicates some of the anti-tumor therapies that are being developed or under consideration. Figure reprinted from (Weinberg, 2007).

The inflammatory microenvironment of tumor cells is mostly constituted of leukocytes. A variety of leukocyte populations have been involved in the promotion of tumor growth and metastasis. These populations include macrophages (the TAM, Tumor-Associated-Macrophages), granulocytes and myeloid suppressor cells. Dendritic cells and lymphocytes are also present in tumor cell infiltrates (Negus et al., 1997). For example, in a mouse model of epithelial carcinogenesis, B lymphocytes were required for establishing chronic inflammatory states that promote de novo carcinogenesis (de Visser et al., 2005). In colorectal cancer, the tumor infiltration by dendritic cells and their localization was correlated with a bad

prognosis for the patient (Sandel et al., 2005). MMP-9 expressing neutrophils were shown to play an important role in the early stages of carcinogenesis in activating angiogenesis (Nozawa et al., 2006).

Tumor-Associated Macrophages (TAM)

The Tumor-associated Macrophages (TAM) represent the major population in tumor infiltrates and have been shown to be implicated in many steps of tumor progression. TAM are the most abundant cells to be recruited by tumor cells and attracted on primary tumor sites by cytokines. They accumulate in hypoxic areas of tumors due to HIF-1 dependent upregulation of the chemokine receptor CXCR4 (Schioppa et al., 2003). They are obligatory partners for tumor metastasis and well studied (Pollard, 2004; Wyckoff et al., 2007). The association of chronic inflammation with cancer together with positive results obtained with anti-inflammatory drugs support the role of TAM in cancer (Balkwill et al., 2005). In addition, it has been shown that a high density of TAM is correlated with a poor prognosis for cancer patients (Bingle et al., 2002; Lin et al., 2002). TAM have a dual role (Mantovani et al., 1992). On the one hand, they can eliminate tumor cells; on the other hand, they can promote tumor progression by producing growth factors for tumor cells or even proteases that would help to degrade the ECM. This last property was illustrated by a study showing that TAM can contribute to skin carcinogenesis by producing MMP-9 (Coussens et al., 2000). The overexpression of colony-stimulating factor-1 (CSF-1) recruiting macrophages in a mouse model of breast cancer leads to tumor progression and metastasis (Lin et al., 2001), whereas the removal of macrophages in the same model by knocking-down the gene coding for CSF-1 dramatically reduced the rate of metastasis. Moreover, macrophages were shown to promote tumor angiogenesis. Indeed, angiogenic switch and the progression to malignancy were shown to be regulated by infiltrated macrophages in a mouse mammary tumor (Lin et al., 2006). Depletion of TAM by clodronate-liposomes leads to a reduction of tumor growth in murine and human models most probably due to the inhibition of angiogenesis (Zeisberger et al., 2006).

Macrophages are clearly associated with tumor development; however their precise function still remains to be defined. How do macrophages help tumor cells to progress at distant sites? What are the underlying mechanisms involved in this process?

The molecular mechanisms linking inflammation and development of carcinomas are being intensively studied (de Visser et al., 2006; Karin, 2006; Luo et al., 2007). The role of NFκB

and its activating IκB kinases were shown to be crucial in the regulation of cell survival and production of pro-inflammatory cytokines. Macrophages enhanced cancer metastasis in the mouse model of prostate cancer by secreting the proinflammatory cytokine receptor activator of NFκB ligand RANKL (Luo et al., 2007). In these primary prostate cancers, RANKL has been shown to be expressed by infiltrating T lymphocytes and macrophages. In addition, it was shown that prostate cancer cells produce higher amount of the chemokine MCP-1 (Monocyte Chemotactic Protein-1), chemokine recruiting leukocytes during inflammation, and that MCP-1 in addition to IL-8 mediates tumor-induced osteoclast activities important for bone metastasis (Lu et al., 2007).

Mice bearing transplantable tumors, but also mice having cancer as a consequence of chemical carcinogenesis undergo a progressive accumulation of myeloid suppressor cells (MSCs) in the spleen and blood. This increase can be taken as an indirect measure of tumor dissemination (Melani et al., 2003). These cells share the CD11b and the Gr-1 markers and are a heterogeneous population, enclosing granulocytes, monocytes and varying numbers of immature cells of the myelomonocytic lineage (Serafini et al., 2004). In the 1980's, a first evidence was found for a correlation between myeloid cell accumulation and immune suppression (Maier et al., 1989; Subiza et al., 1989). In a mouse adenocarcinoma model, it was reported that TAM can induce apoptosis in activated T cells by a mechanism requiring cell contact, TNF-α, and NO production (Saio et al., 2001; Serafini et al., 2004).

Accumulating evidences strongly support the decisive role of macrophages associated with tumor cells in the contribution to tumorigenesis. Growth factors produced by macrophages support the tumor cell microenvironment and stimulate angiogenesis.

2 Cell-cell interactions during metastasis

2.1 Metastasis

Metastasis is defined as spread of primary tumors to a distant organ. These distant settlements of tumor cells are the cause of 90 % of human cancer death (Sporn, 1996). The spread of metastases can occur via the blood or lymphatic vessels or through both routes.
In 1889, Stephen Paget has proposed that metastasis is not random but a cross-talk between the tumor cells as 'seeds' and a specific organ microenvironment as 'the soil' (Paget, 1889). This 'seed an soil' theory suggested that the destiny of tumor cells was not by chance but that tumor cells form metastases only in organs where the 'soil' is favorable (Fidler, 2003). In 1929, James Ewing challenged this theory by the affirmation that metastasis occurs only by mechanical factors resulting from the anatomical structure of the vasculature. But Paget's theory is nowadays considered to be the closest to the reality (Liotta and Kohn, 2001).
Metastasis is a very inefficient mechanism, because most of the tumor cells that enter the bloodstream are rapidly eliminated (Fidler, 2003). Less than 0.05 % of circulating tumor cells are successful in initiation of metastatic colonies. Metastasis formation is a highly selective process, which contradicts a widely accepted belief that metastasis represents the ultimate expression of cellular anarchy. It has been shown that primary tumors influence the environment in the lungs before metastasis. Tumors secrete VEGF-A, TGFβ and TNFα, which induce expression of chemokines by myeloid cells and endothelial cells within the lungs before metastasis and this chemokine-activated premetastatic niche supports adhesion and invasion of malignant cells (Hiratsuka et al., 2002; Kaplan et al., 2005).

In the present work we focused on the mechanism of hematogenous metastasis. The tumor cells first acquire the capacity to degrade and breach the basement membrane and invade blood vessels in a process called intravasation (Weinberg, 2007). They have then to survive in the circulation which is a hostile environment for metastasizing cancer cells due to the presence of innate immune cells. Indeed, they have to escape the clearance by immune cells, they are threatened by dying from anoikis and they have to survive the hydrodynamic shear forces in the circulation. The survival of these cells has been shown to be enhanced by

aggregation of platelets around them (Nieswandt et al., 1999; Palumbo et al., 2005). Tumor cell interaction with platelets and leukocytes, leading to the formation of a microembolus facilitates their arrest in the vasculature (Honn et al., 1992b; Karpatkin et al., 1988; Kim et al., 1998). The cells finally arrest in a new organ and extravasate from the circulation into the surrounding tissue **(Figure 4)**.

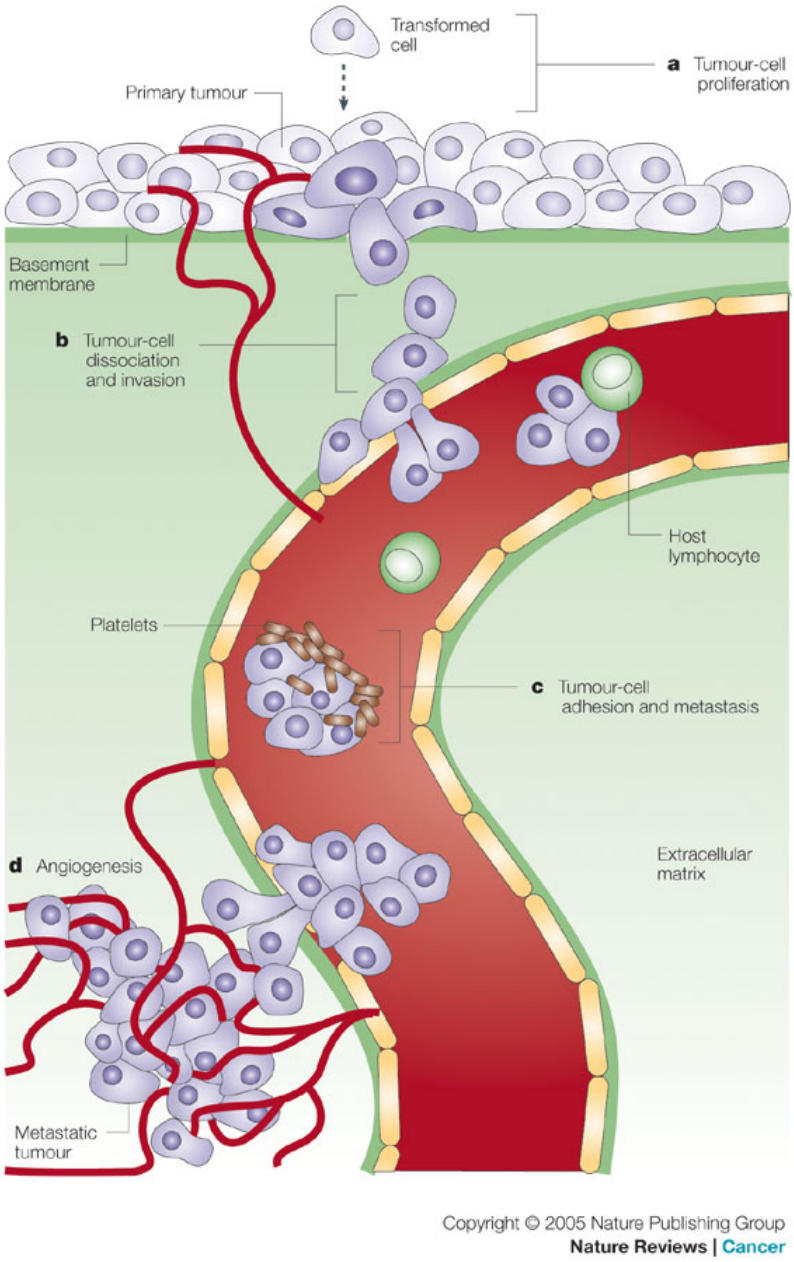

Figure 4: *Metastasis is a multi-step process.* Figure reprinted from (Fuster and Esko, 2005).

Capillaries have diameter of 3-8 µm allowing the passage of red blood cells which are about 7 µm in diameter and highly deformable but not allowing the passage of tumor cells which are

much bigger (20 μm) and not so deformable (Chambers et al., 2002). The coating of the tumor cells by platelets further increases their diameter, causing them to be trapped also in larger vessels. Structural and molecular basis for the arrest of such emboli in the vasculature and for the formation of a new metastatic site remains to be fully elucidated. For example, pulmonary arrest does not occur merely due to size restriction of the capillaries but the early arrest of tumor cells in the pulmonary vasculature can be mediated through interaction of α3β1 integrin with laminin-5 in exposed basement membrane. (Wang et al., 2004). The role of platelets and leukocytes in tumor cell entrapment in the vasculature has been extensively studied (Borsig et al., 2002; Honn et al., 1992a; Karpatkin et al., 1988). Platelets, leukocytes and tumor cells form tumor cell emboli in the vasculature leading to an enhanced tumor cell progression. The platelet aggregation around tumor cells protects the tumor cells from the native immune system and facilitates the arrest and extravasation of tumor cells at new metastatic sites (Borsig et al., 2002; Kim et al., 1998).

2.2 Alteration in tumor cell surface glycosylation and tumor progression

Alterations associated with malignancy

Many alterations are associated with carcinoma malignancy. Tumor progression involves alterations in both intracellular and extracellular signaling. In order to metastasize, cells need to be able to detach from other cells and from the substrate by alteration of cell-adhesion molecules, like cadherin, and in cell surface glycosylation. Tumor cells need then to degrade the extracellular matrix by releasing matrix metalloproteinases, like MMP-9 and to migrate through host ECM and basement membranes. Further progression needs recruitment of an endothelial-lined neovascular network from endothelial cells for angiogenesis, to acquire cell-surface characteristics that promote adhesion with platelets, leukocytes and endothelial cells and to escape innate immunity. Finally, metastases are formed from cells that survive.

O-Glycan alteration during tumor progression

Several glycans on the tumor surface have now been identified as mediating key events during the various steps of tumor progression (Fuster and Esko, 2005). During the process of tumorigenesis, the cells undergo a transformation called epithelial-mesenchymal transition (EMT). During EMT, epithelial cells adopt a migratory phenotype and this transition is recognized as a pathological process contributing to cancer progression (Arias, 2001). During this process, epithelial cells lose their polarity, and mucins expressed all over the cell block cell-cell and cell-substratum adhesion through a mechanism of repulsion **(Figure 5)**.

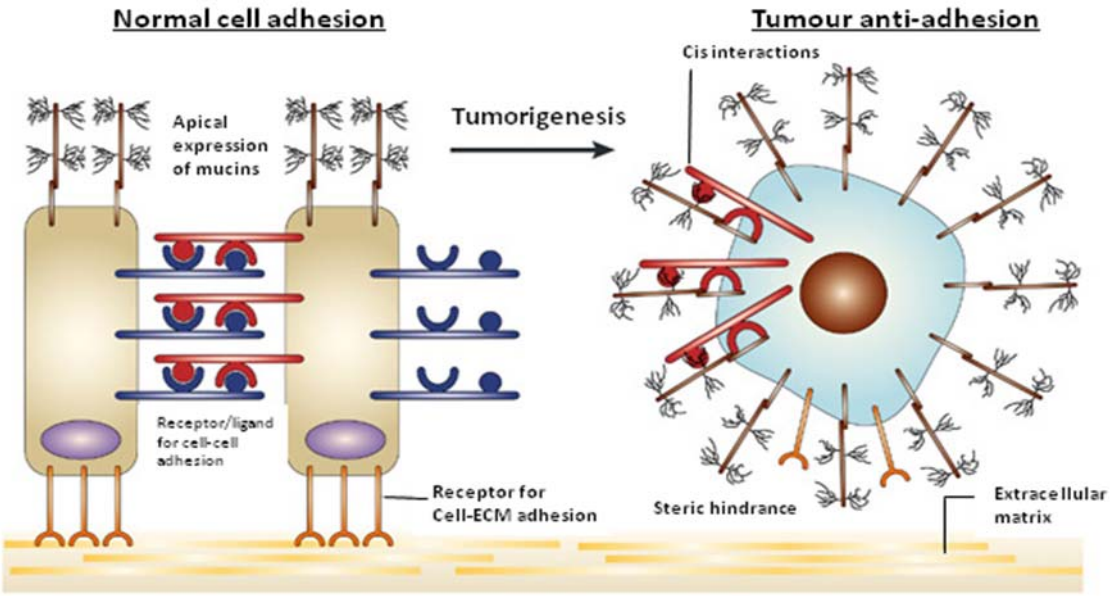

Figure 5: *Tumorigenesis and loss of apical expression of mucins.* During EMT, epithelial cells lose their polarity, and mucins are aberrantly expressed, blocking cell-cell and cell-substratum adhesion. Figure reprinted from (Hollingsworth and Swanson, 2004).

O-linked glycans are extremely diverse in both structure and function and are built on protein-glycan linkages, in which sugar structures like GalNAc, fucose, mannose or galactose can be attached to seronine, threonine or hydroxylysine residues. Mainly two groups of glycoproteins, mucins and proteoglycans are dominated by the large number of O-linked glycans they bear. During EMT, epithelial cells lose their polarity and do not express mucins on their apical surface anymore **(Figure 5)**. Mucins, highly O-glycosylated proteins have an important role in maintaining homeostasis for epithelial cells living in variable environment. In comparison with O-glycans from normal mucin, O-glycans from cancerous mucins can be highly sialylated and less sulphated (Brockhausen, 2006). The metastatic potential of tumor cells has been correlated with increasing sialylation of cell-surface glycoproteins (Taylor and Drickamer, 2006). The O-glycans can also be truncated and contain the Tn (GalNAc-) and T (Galβ1-3GalNAc-) antigens **(Table 3)**.

***O*-glycan Increased**	**Structure**	Increase / decrease in cancer*
Tn antigen	. GalNAcα-Ser/Thr	↑
STn antigen	. Sialylα2-6GalNAcα-Ser/Thr	↑
Core 1, antigen	. Galβ1-3GalNAcα-Ser/Thr	↑
Sialyl-T antigens	. Sialylα2-3Galβ1-3GalNAcα-Ser/Thr	↑
	. Sialylα2-6(Galβ1-3)GalNAcα-Ser/Thr	↑
Core 2	. GlcNAcβ1-6(Galβ1-3)GalNAcα-Ser/Thr	↑↓
Core 3	. GlcNAcβ1-3GalNAcα-Ser/Thr	↓
Core 4	. GlcNAcβ1-6(GlcNAcβ1-3)GalNAcα-Ser/Thr	↓
Type 1 chain	. [GlcNAcβ1-3 Galβ1-3]$_n$	↓
Type 2 chain	. [GlcNAcβ1-3 Galβ1-4]n	
	. Poly-N-acetylactosamines	↑
Sialyl-Lewis^a	. Sialylα2-3Galβ1-3 (Fucα1-4)GlcNAcβ1-3Gal-	↑
SLe^x	. Sialylα2-3Galβ1-4 (Fucα1-3)GlcNAcβ1-3Gal-	↑
Sialyl-dimeric Lewis^x	. Sialylα2-3Galβ1-4 (Fucα1-3)GlcNAcβ1-3 Galβ1-4 (Fucα1-3)GlcNAcβ1-3Gal-	↑

Table 3*:* *Mucin-type O-glycans and alterations in cancer.* *The symbol ↑ denotes an increase in cancer, whereas the symbol ↓ denotes a decrease. Table adapted from (Brockhausen, 2006).

These alterations of glycosylation with aberrant terminal sugar structures of O-glycan chains, such as Lewis antigens and sialic acids, play an important role in the biology of cancer. Indeed, the interactions between cancer cells and their microenvironment can be mediated through sugar binding molecules, like lectins. The number of sialyl-Lewis^x (SLe^x) epitope, tetrasaccharide, characterized by the presence of terminal fucose and sialic acid residues, is correlated with a poor survival of patients with colon cancer (Nakamori et al., 1993) **(Figure 6)**.

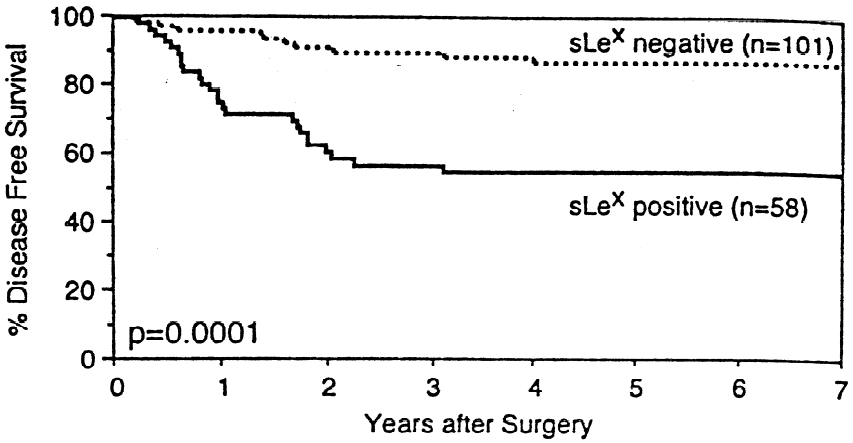

Figure 6 : *The number of sialyl-Lewis^x (SLe^x) epitope is correlated with a poor survival of patients with colon cancer.* Figure reprinted from (Nakamori et al., 1993).

Compared with primary tumors, metastatic cancer cells have often increased amount of sLe^x and sLe^a, as well as sialyl-dimeric Lewis^x (Ito et al., 1997) **(Figure 7-A)**. These structures are normally involved in the attachment of leukocytes to endothelium via selectin molecules, during an inflammatory process, for example. Overexpression of glycosylated structures is not the only alteration associated with carcinoma malignancy. Some glycans can be truncated, like the sialyl-Tn epitope / antigen, which is a truncated O-linked glycans and exposed at high levels on a variety of carcinomas **(Figure 7-B)**. O-glycans can be truncated *in vitro* by inhibiting glycosyltransferases.

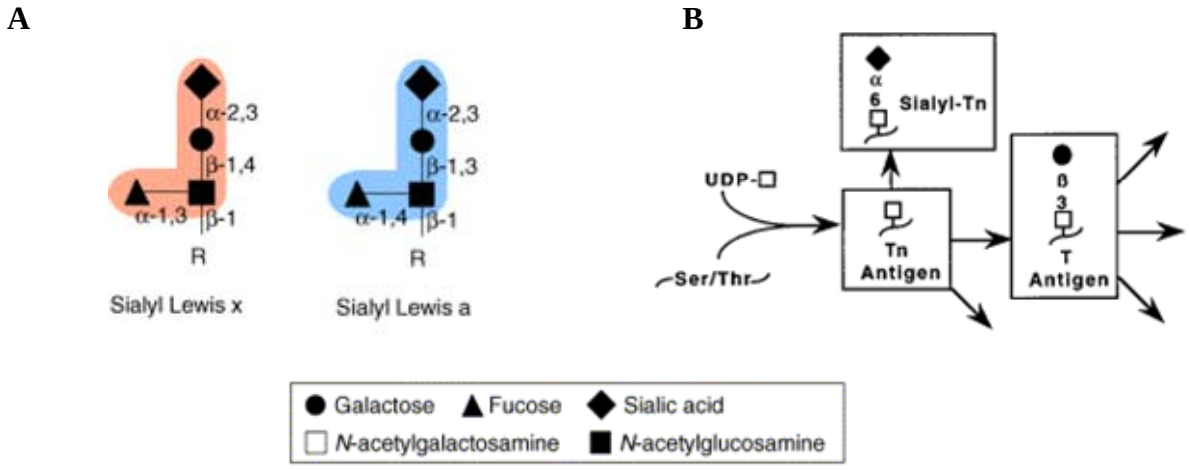

Figure 7: *Glycosylated structures involved in carcinoma malignancy:* **A** *Sialyl Lewis^x and ^{a}.* Expression of these structures by cancer cells is linked with carcinoma malignancy. Figure reprinted from (Lowe, 2003). **B** *Tn and T antigen*. Incomplete glycosylation in the O-linked pathway results in expression of the Tn antigen, the sialylated Tn antigen (a "dead-end" structure), or the T antigen (Thomsen-Friedenreich antigen, or unmodified Core 1 structure). Multiple copies of these structures may occur in a closely spaced array on the polypeptide. Figure reprinted from (Varki et al., 1999).

O-linked oligosaccharide synthesis was inhibited by benzyl-alpha-N-acetylgalactosamine -benzyl-alpha-GalNAc- (Kuan et al., 1989). Benzyl-alpha-GalNAc is a substrate decoy molecule for GalNAc transferase and can therefore reduce the glycosylation of cell-surface glycoproteins. The accumulation of O-linked αGal-NAc at the surface of the cells, resulting from this blocking can be quantified through increased reactivity with FITC-labeled *Helix pomatia* lectin. The use of this inhibitor on colon cancer cells reduces the mucin-type O-glycans as well as their attachment to E-selectin and endothelial cells (Kojima et al., 1992). In addition, tumor cell treatment with an acetylated disaccharide, inhibitor of the biosynthesis of sLe^x in a model of spontaneous metastasis in mice attenuates metastasis *in vivo* (Brown et al., 2006), suggesting that alteration of O-glycosylation has a direct effect on invasion and metastasis.

A study of the binding of colon cancer cells with purified selectins showed that L- and P-selectin interact with mucin-type ligands on colon cancer cells whereas E-selectin can recognize both mucin and non-mucin ligands (Mannori et al., 1995). This finding correlates with the hypothesis that colon cancer cells adhere to activated endothelium (E-selectin dependent), platelets (P-selectin dependent) and leukocytes (L-selectin dependent).

Furthermore clustered presentation of sLe^x on tumor cell mucins is suggested to facilitate metastasis through binding to selectin on platelets, leukocytes and endothelial cells (Kim et al., 1999).

Changes in glycosylation are due to an up- or down-regulation of the glycosyltransferases or their mislocalization in the secretory pathway of tumor cells. Many tumor cells have altered mRNA levels and activities of glycosyltransferases. In human colon adenocarcinoma of high grade, the type 2 chains, precursor for the sLe^x antigen, are produced and the activity of β4-Gal-transferase involved in their synthesis is upregulated **(Table 3)**. Increased activities of α-2,3-sialyltransferases leading to an increased amount of sLe^x in colon carcinomas have also been detected (Majuri et al., 1995).

Carcinomas commonly express O-linked glycans that present ligands for selectins, promoting tumor cells interaction with platelets, leukocytes and endothelial cells. These interactions are suggested to facilitate haematogenous metastasis of tumor cells.

Tumor-associated antigens

Tumor-associated antigens (TAAs) on several mucins have an oligosaccharide structure and some correspond to blood-group antigens (Piller et al., 1980). Carcinoma mucins are secreted in the circulation and can be used as markers. Serological markers like CA125, CA19-9 and CA15-3 are mucin glycoconjugates commonly overexpressed by ovarian, pancreatic and breast adenocarcinomas, respectively. Serum levels of these TAAs correlate with tumor burden and poor prognosis. Antibodies against the TAAs are used as diagnostic tools in serum assays. For instance, the mAb CA19-9, recognizing sLe^a on pancreatic carcinoma mucins is used for the detection of colorectal and pancreatic adenocarcinoma (Hayashi et al., 2004). Oncofetal antigens like carcinoembryonic antigen (CEA) and the breast cancer MUC-1 are overexpressed in tumors but can be expressed in other tissues as well. Since these antigens are also found in fetal tissues, it is not so easy to use them as therapeutic targets. Indeed, peripheral tolerance to these antigens can exist and a vaccination could lead to autoimmune reactions.

N-glycans and tumor progression

Tumor progression associated with changes in glycosylation is not only due to changes in O-glycans; it has been shown in a model of breast cancer, that tumor progression is associated

with increased β-1,6-GlcNAc-branching, a regulatory step in expression of polylactosamine and extended-chain Lewis antigens. This increased sialylation and increased branching of complex N-linked oligosaccharides in metastatic cells results from an increased activity of GlcNAc-transferase V (GnTV). For example, the presence of complex β-1,6-branched N-glycans on tumor-cell E-cadherin -an adhesion molecule that normally mediates cell aggregation - reduces tumor cell–cell adhesion. Therefore, increased expression of this enzyme promotes cell detachment and invasion (Dennis et al., 2002; Fuster and Esko, 2005; Yoshimura et al., 1996). Interestingly, E-cadherin is often downregulated by invasive cancers (Hirohashi and Kanai, 2003).

In addition, increased β-1,6-branching on the β1 subunit of tumor α5 β1 integrins, disrupts the ability of integrins to cluster on the tumor-cell membrane (Guo et al., 2002) This altered integrin clustering reduces the formation of tumor-cell focal adhesions, and increases tumor motility through the ECM as well as invasion across basement membranes.

Treatment of the cells with Swainsonine and other inhibitors of N-linked oligosaccharide synthesis, blocks tumor cell invasion *in vitro* and reduces tumor growth *in vivo* (Kim and Varki, 1997; Korczak et al., 1994).

Tumors aberrantly express various glycans that regulate many different aspects of tumor progression. Investigating the role of O-linked glycans in tumor progression is one of the aims of our study. Tumor mucins present ligands for various cell adhesion molecules, such as selectins, that can mediate the interactions with platelets, leukocytes and endothelial cells. These interactions may facilitate haematogenous metastasis of tumor cells.

2.3 Selectins and their physiological functions

Lectins are proteins or glycoproteins which recognize carbohydrate structures. They have diverse functions, being implicated in innate immunity (for example the mannose-binding protein MBP), in antigen presentation (C-type lectin), in adhesion (selectins) and signaling events (galectins, I-type lectins like siglecs) or in T-cell apoptosis (galectin-3, -1 and -9). Many lectins can potentially recognize the altered glycosylation of cancer cells (Kim and Varki, 1997). For example, galectins (especially galectin-3) can interact with poly-N-acetyl-lactosamines on laminin, helping invasion and could bind also polylactosamines expressed on cancer mucins (Inohara et al., 1996). A complement regulatory protein, the H protein and I-type sialic acid binding proteins like CD22 are also potential helpers in the invasion mechanism (Kim and Varki, 1997).

More particularly, the selectins, which have important physiological functions in cell adhesion mediating leukocytes rolling on endothelium in response to inflammation, have shown to be active players in cancer. We are studying carcinoma cells with altered glycosylation, expressing sialylated fucosylated mucins that can interact with platelets, leukocytes and endothelium *via* the selectin family of cell adhesion molecules.

Selectin family

Selectins are transmembrane C-type lectins that bind carbohydrates in a calcium dependent manner (Kansas, 1996). They are cell-adhesion molecules for leukocytes, playing a role in the homing of T- and B- cells to peripheral lymph nodes and in the rolling of leukocytes on endothelium for leukocytes migration to inflammation sites (Kansas, 1996; McEver, 2002).

There are three types of selectins: L-, P- and E-selectin, that share a very similar structure; a carbohydrate-binding domain at the N-terminus, an EGF-like domain, various numbers of consensus repeats with homology to complement regulatory proteins, a transmembrane domain and a cytoplasmic tail involved in signaling **(Figure 8)**.

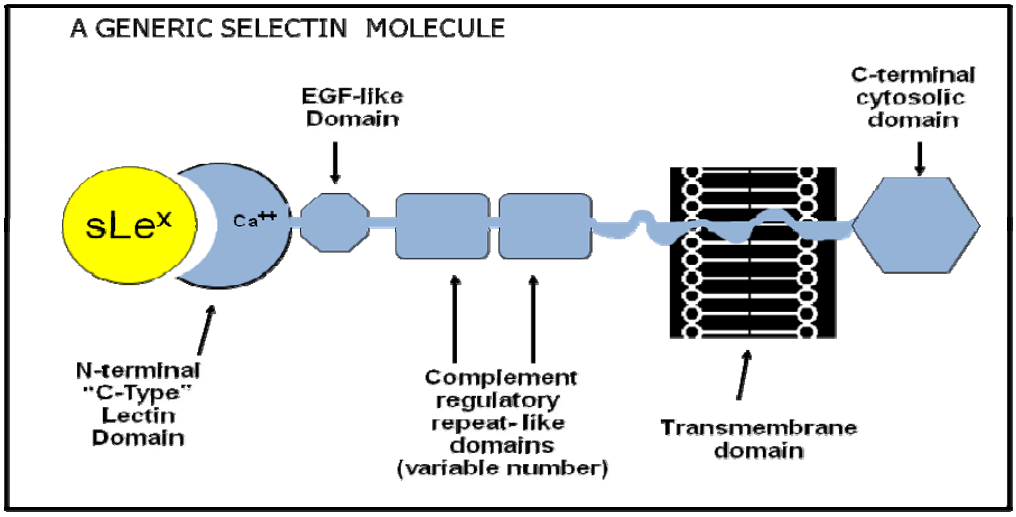

Figure 8: *A generic selectin molecule binding a common ligand: sLe^x.* There are three types of selectins: L-, P- and E-selectin, that share a very similar structure.

L-selectin is constitutively expressed on the surface of leukocytes, E-selectin on activated endothelium and P-selectin is rapidly exteriorized under activation of platelets or endothelium. L-selectin mediates the binding of leukocytes to endothelial cells in the lymph nodes. P- and E-selectins help the migration of the neutrophils into sites of inflammation. During inflammation, endothelial cells are activated and express P-selectin stored in Weibel-Palade bodies (Dole et al., 2005) to their cell surface thereby recruiting circulating neutrophils. L-selectin causes the attachment of the attracted leukocytes to the endothelium.
L-selectin ligands on high endothelial venules (HEV) are sialomucins like GlyCAM-1 (mouse), CD34 (mouse and human), podocalysin (human), endomucin (member of the PNAd complex) and MadCAM-1 (also member of the PNAd complex). These sialomucins are functionally redundant. This situation is totally different from the case of P-selectin, indeed its ligand is a single sialomucin called PSGL-1. PSGL-1 is the major leukocyte ligand for both P- and L-selectin (Rosen, 2004) and interestingly, PSGL-1 has been shown to mediate L-selectin-dependent leukocytes rolling in venules (Sperandio et al., 2003).

The diapedesis of leukocytes in inflammation is selectin dependent

Leukocytes are extravasating the circulation to enter into the parenchyma in response to inflammatory stimuli in a process called diapedesis. The leukocytes are able to induce the retraction of the endothelial cells and the process from attachment to the endothelial wall till the entry into the tissue takes less than a minute (Varki et al., 1999).

This trafficking of leukocytes in postcapillary venules is a multi-step mechanism which involves their initial attachment to the endothelial cells, followed by rolling on the endothelial cell wall and finally arrest and transmigration through the endothelial cell layer **(Figure 9)**. Rolling characterized by a weak adhesion is mostly selectin-mediated (L-selectin on the surface of the lymphocytes, E- and P-selectin on endothelial cells), whereas the strong adhesion and arrest is integrin-mediated (intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM-1). The dimerization of L-selectin leads to stabilization of cell rolling. Indeed, glycan-lectin interactions require clustering of lectin domains to generate avidity between the corresponding components (Weis and Drickamer, 1996). Inflammatory response is accompanied by an activation of the neutrophils. Briefly, neutrophils first roll *via* selectin interactions and adhere to vascular endothelium, mobilize secretory vesicles, enrich their surface with the β2-integrin CD11b/CD18 and shed L-selectin. They then extravasate and degranulate and are able *in situ* to eliminate microorganisms for example. *In vitro*, the stimulation with inflammatory mediators leads to a rapid release of secretory vesicles and granules.

Leukocyte trafficking is affected in mice deficient in selectins (Tedder et al., 1995). The lymphocytes of L-selectin deficient mice were not able to bind the peripheral lymph node high endothelial venules -HEV- (Arbones et al., 1994). The mutant mice had a severe reduction of the number of lymphocytes localized to the lymph nodes. This model shows the important role of L-selectin in leukocyte homing to lymphoid tissues and in inflammation.

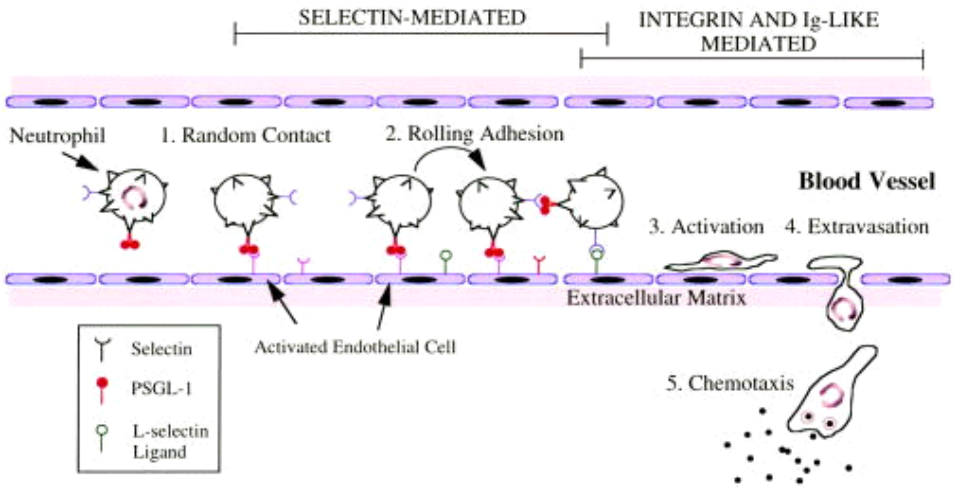

Figure 9: *Rolling adhesion of circulating leukocytes to activated endothelium via interactions between selectins and their ligands.* This figure shows a neutrophil expressing PSGL-1 and L-selectin and interacting with membrane P-selectin on endothelial cells. E-selectin may also participate in these interactions by binding to PSGL-1 or other ligands on these cells. Following interactions, cells are tethered and roll along the endothelium. Leukocyte-leukocyte interactions involving L-selectin and PSGL-1 are depicted, but L-selectin may also have other ligands expressed by the activated endothelium. Adherent cells become activated by regionally presented chemokines. The activated leukocytes express integrins that interact with Ig-like counter receptors on endothelial cells, strengthen the adhesion, and promote the emigration or extravasation (diapedesis) of cells into the underlying tissues in response to chemotactic gradients. Figure reprinted from (Varki et al., 1999).

2.4 Heterotypic interactions during metastasis

Lymphatic and blood vessels are the major anatomical pathways for the dissemination of tumor cells. Endothelial cells and the vessels that they form ensure tumors adequate access to the circulation. Within the vasculature the tumor cells interact with endothelial cells, platelets and leukocytes. The importance of aggregation of tumor cells with cellular blood components for metastasis is now well established (Borsig et al., 2001b; Honn et al., 1992b; Karpatkin et al., 1988)

2.4.1 Endothelium and metastasis

Tumor cells are associated with the vasculature to access to nutrients and oxygen

Endothelial cells are highly metabolically active and play a role in many physiological functions, like blood cell trafficking, hemostatic balance, permeability and immunity (Aird, 2003). Tumors require access to the circulation in order to grow and survive. As early as the mid-1950s, pathologists realized that tumor cells preferentially grew around blood vessels. It has been shown that oxygen diffusion was one important explanation why tumor cells were so close to blood vessels. Indeed, tumor cells need effective interactions with the vasculature in order to acquire nutrients and to shed metabolic waste and carbon dioxide. Tissues suffering from hypoxia are in danger of becoming apoptotic by activation of p53-signaling. Inactivation of the p53-signaling system often enables tumor cells to survive beyond the small perimeter surrounding each capillary. The formation of blood capillaries during angiogenesis involves a high number of factors, like VEGF, TGF-β, basic fibroblast growth factors (bFGFs), interleukin-8 (IL-8), angiopoietin, angiogenin and PDGF. Moreover, mural pericytes and vascular smooth muscle cells help this process as well.

Interactions endothelium-cancer cells in metastasis

The arrest of circulating cancer cells in the vasculature is a prerequisite for their extravasation and growth into metastatic tumors. Mechanical factors alone are not sufficient for the ultimate

tumor cell arrest in target organ circulation, and specific adhesive interactions between metastatic cells and blood vessel endothelia are necessary for malignant cell arrest in microvessels. Indeed, many metastatic cells arrested in lung circulation, reside in precapillary arterioles of calibers far exceeding tumor cells in size (Wong et al., 2002). Adhesive factors and arrest specificity promote metastasis (Orr et al., 2000). The adhesion between cancer cells and endothelium is selectin-, integrin-, cadherin- or immunoglobulin-dependent. Indeed, *in vitro* metastatic breast and prostate carcinoma cell adhesion to microvascular endothelium is mediated largely by interactions between cancer-associated Thomsen-Friedenreich (TF) glycoantigen (Galβ1–3GalNAc) and β-galactoside-binding lectin galectin-3 (Glinsky et al., 2001). Similarly, it has been shown that sLe^a and sLe^x, respectively on colon carcinomas and renal carcinomas bind the adhesion molecule E-selectin on endothelial cells (Lafrenie et al., 1994; Steinbach et al., 1996; Ye et al., 1995). These facts demonstrate unambiguously that blood-borne malignant cells could be arrested in a variety of organs and tissues through specific adhesive interactions with vessel walls in the absence of mechanical entrapment.

Some studies even describe a loss of endothelial barrier integrity during tumor cell transmigration (Heyder et al., 2002; Weis et al., 2004). Vascular permeability is characterized by altered cell-cell contacts and the appearance of paracellular pores between adjacent cells. Integrity of the endothelial barrier is regulated in part by opposing roles of the actin cytoskeleton in which cortical F-actin stabilizes cell-cell contacts, whereas intracellular stress fibers exert tension to induce permeability (Stockton et al., 2004).

2.4.2 Platelets and metastasis

Circulating platelets contribute to haemostasis, thrombosis and inflammation. The presence of platelets in association with cancer has first been recognized by the surgeon Billroth in 1878, who described the association of cancer cells with a thrombus -plug of platelets and blood constituents- (Nash et al., 2002). In this context, an artificial decrease in platelet count leads to an attenuation of metastasis due to an enhanced clearance of tumor cells (Gasic, 1984). Thrombin is a well-known platelet activator and can be derived from tumor cells. The primary role of thrombin is the conversion of fibrinogen to fibrin, the building block of a hemostatic plug. When platelets are activated by thrombin, there is a rapid signal transduction cascade resulting in massive influx of Ca^{2+} into the platelets cytosol. This leads to a cross-linking of the actin filaments of the platelet that becomes activated and extend lamellipodia and filopodia and spread itself across the clot. Thrombin released by tumor cells leads to platelet activation and their adherence to tumor cells (Nierodzik and Karpatkin, 2006). Platelets that accumulate on tumor cells prolong tumor survival in the circulation by impeding their elimination by natural killer cells (NK cells) and facilitate their arrest and adhesion on endothelial cells, thereby helping them to extravasate and metastasize (Nieswandt et al., 1999; Palumbo et al., 2005). Platelets also secrete tumor cell growth and angiogenesis factors, such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) which leads to vascular hyperpermeability (Poggi et al., 1993). Platelet-derived lysophosphatidic acid (LPA) promotes tumor cell proliferation by binding the LPA_1 receptor of breast and ovarian cancer cells (Boucharaba et al., 2004). Cancer patients have generally higher circulating concentrations of VEGF than healthy persons (Poon et al., 2001). Tissue factor, upregulated at hypoxic sites of tumor tissue leads to platelet activation as well. Prostacyclin, synthesized by vascular endothelial cells inhibits the action of procoagulant thromboxane A_2 inducing platelet aggregation. The ratio prostacyclin to thomboxane A_2 concentrations is disturbed in many cancers and endothelial cells deficient in prostacyclin showed an increased tumor-endothelial adhesion (Honn and Tang, 1992). Inhibition of platelets by prostacyclin and anticoagulants are used to inhibit tumor metastasis and these antiplatelets agents have some anticoagulant activities. It has been shown in our group that heparin decreases metastasis in mice by blocking P-selectin-mediated-platelet interactions with tumor cell surface mucins (Borsig et al., 2001a). Low anticoagulant heparin preparations still inhibited metastasis efficiently, indicating that anticoagulation is not a necessary component for heparin attenuation of metastasis (Hostettler et al., 2007).

2.4.3 Leukocytes and metastasis

Increasing evidence indicates that leukocytic infiltration can either impede tumor formation and growth, in a process called immunosurveillance (Dunn et al., 2004) or, alternatively, promote tumor phenotypes, such as angiogenesis, growth and invasion, in a process called immune enhancement (de Visser et al., 2006; de Visser et al., 2005). Clinical data for solid tumors show a correlation between high-density leukocytic infiltration into tumors and poor outcome for patients (Coussens and Werb, 2002).

Neutrophils are the most abundant circulating blood leukocytes in humans. They are the first cells recruited to inflammation sites. They have a 'natural' antitumor activity and are able to produce many cytotoxic compounds, such as reactive oxygen species, proteases, membrane-perforating agents and soluble factors like TNF-α, IL-1β and IFNs (Di Carlo et al., 2001). On the other hand, they can promote tumor progression. Indeed, neutrophils were shown to enhance the metastatic potential of tumor cells in many carcinoma models (Queen et al., 2005; Tazawa et al., 2003; Welch et al., 1989). In an *in vitro* transendothelial model, human neutrophils were assisting a human breast tumor cell line to cross the endothelial barrier, suggesting an important role of the neutrophils in the tumor cell extravasation (Wu et al., 2001). There are, in fact, two possible ways how neutrophils assist tumor cell migration through the endothelial barrier. The first possibility is that the neutrophils cause endothelial injury by releasing proteases and reactive oxygen species thereby causing microvascular permeability. This has been seen in a tumor-bearing murine model in which neutrophils-mediated pulmonary injury facilitated metastasis in the lung (Orr and Warner, 1990). The other possibility is an interaction between neutrophils, highly transmigratory cells, and tumor cells, leading to a co-migration of the tumor cells through the endothelium.

2.4.4 Selectin and metastasis

Selectin molecules on platelets, leukocytes, and endothelium promote metastasis

Tumors carry altered cell surface glycosylation containing sLe^x and sLe^a, both epitopes recognized by selectins. The expression of these epitopes is correlated with tumor progression and metastasis in human (Nakamori et al., 1993). In addition, human carcinoma cell lines, like LS180, express sialomucins that can bind L- and P-selectins (Mannori et al., 1995). All three selectins were able to bind to carcinoma cell lines in a calcium dependent manner (Mannori et al., 1995) and this recognition was abolished by O-sialoglycoprotease, a mucin endopeptidase (Borsig et al., 2001a). Mucins had separate binding sites for each selectin, allowing cross-binding of a mucin molecule by more than one selectin. Leukocytes, platelets and endothelial cells can then interact with tumor cells at the same time (Kim et al., 1999). In this project, we were mostly interested in the role of L-selectin during cancer progression.

E-selectin expressed by endothelial cells and metastasis

Sialyl-Lewis x/a structures were shown to be specifically recognized by E-selectin and studies describe the presence of E-selectin ligands on carcinoma cells (Majuri et al., 1995). The presence of mucin cancer antigen binding to E-selectin has been detected in the blood of colon carcinoma patients (Zhang et al., 1994). Moreover, the metastatic potential of colon carcinoma cell lines in nude mice increased with the expression of sLe^x (Izumi et al., 1995). Interestingly, overexpression of E-selectin in the mouse liver redirected metastasis of carcinomas that normally colonize the lungs (Biancone et al., 1996). E-selectin is a major mediator for facilitation of metastasis.

P-selectin expressed by platelets and metastasis

Carcinomas bind to activated platelets expressing P-selectin (Mannori et al., 1995) and in P-selectin deficient mice, metastasis is attenuated (Kim et al., 1998). This P-selectin-dependent facilitation of human carcinoma metastasis in immunodeficient mice is due to interaction of platelets with mucins borne by the tumor cells and this process can be blocked by heparin (Borsig et al., 2001a). Studies in immunocompetent mouse gave the same results in terms of attenuation of metastasis but the ligand was shown to be a sulfatide on tumor cells (Borsig et al., 2002; Garcia et al., 2007).

L-selectin on lymphocytes surface and metastasis

The contribution of L-selectin to metastasis has recently been observed. In the absence of L-selectin, metastasis of human adenocarcinoma cells in immunodeficient mice was attenuated (Borsig et al., 2002) **(Figure 10)**. This result was unexpected because expression of L-selectin ligands was thought to provoke tumor cell elimination by leukocytes (Kim and Varki, 1997).

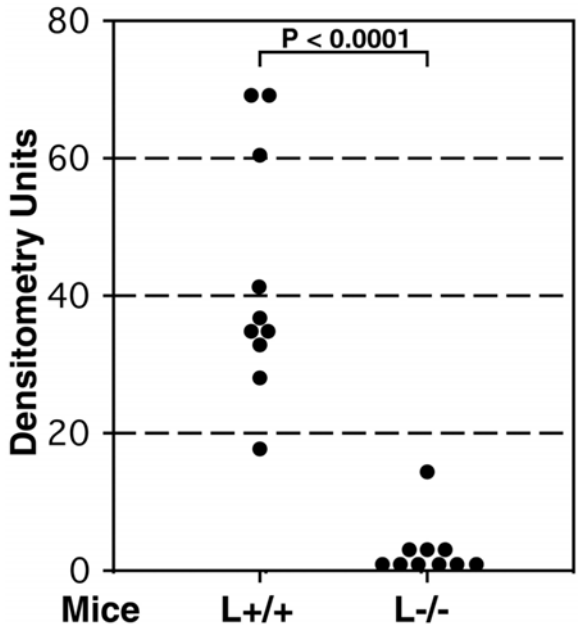

Figure 10: *L-selectin deficiency attenuates metastasis of human adenocarcinoma cells in immunodeficient mice.* Figure reprinted from (Borsig et al., 2002).

In the P- and L- double selectin deficient mice, metastasis was further attenuated in comparison to single deficient mice (Borsig et al., 2002). Thus, contribution of P- and L-selectin seems to be synergistic during metastasis formation. L-selectin promotes recruitment of leukocytes to tumor cells and their survival following their entrance in the vasculature. This indicates a local involvement of leukocytes in establishment of metastatic foci. Moreover, in L-selectin deficient mice, association of Mac-1+ leukocytes with tumor cells was reduced and tumor survival diminished (Läubli et al., 2006) **(Figure 11)**.

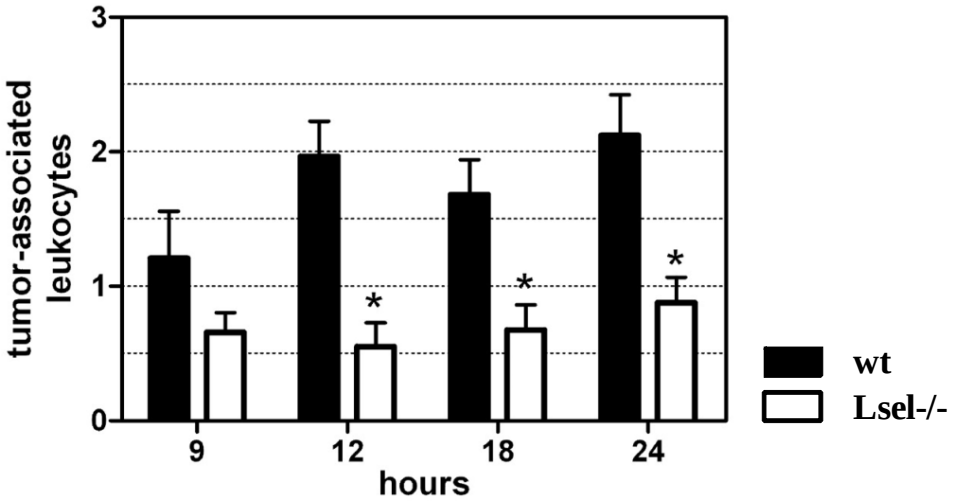

Figure 11: *Association of tumor cells with leukocytes in the wt and L-selectin deficient models.* Figure reprinted from (Läubli et al., 2006).

L-selectin is constitutively expressed by most leukocytes (Kansas, 1996). The role of leukocytes in the facilitation of metastasis by L-selectin seems to be downstream from P-selectin mediated interaction of platelets with tumor cells. The effect of L-selectin was observed even in immunodeficient mice, indicating that granulocytes and/or monocytes are likely implicated in this metastatic process. Leukocytes may bind platelet-carcinoma emboli through L-selectin interactions **(Figure 12)**. This leukocyte-tumor cell association could promote the extravasation by two means: 1) by increasing the size of the tumor embolus which facilitates its mechanical trapping in the microvasculature, 2) by helping tumor cells to extravasate in a L-selectin dependent manner. This second possibility for the observed contribution of leukocytes to metastasis is suggested by the capacity of L-selectin-positive leukocytes to transmigrate through activated endothelium expressing L-selectin-ligand (Hickey et al., 2000; Ley, 2002; Worthylake and Burridge, 2001).

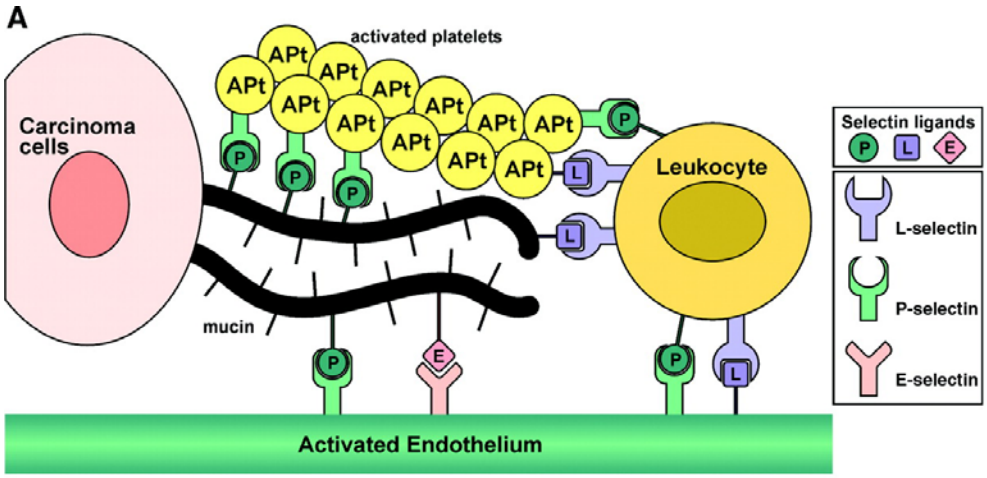

Figure 12: *Possible selectin-tumor cell interactions during malignancy.* Hypothetical model showing all the interactions that can take place between carcinoma cells carrying selectin ligands and selectins expressed on activated platelets, activated endothelial cells and leukocytes. All these interactions have been shown to occur *in vitro* and some have been seen *in vivo*. Figure reprinted from (Borsig, 2004).

3 Aim of the project

Alteration of tumor cell-surface glycosylation is correlated with enhanced metastasis in patients with colon adenocarcinoma. Metastasis is facilitated by the tumor cell embolus formation consisting of blood platelet aggregates and leukocytes around the tumor cells. Carcinomas expressing sLe^x carbohydrate structures can potentially bind P- and L-selectins found at the surface of platelets and leukocytes respectively. The adhesion of platelets to tumor cells is primarily mediated by P-selectin as previously shown. The observation that metastasis was attenuated in the absence of L-selectin implicates leukocytes to be actively involved in the facilitation of metastasis.

The aim of this project was to investigate the nature and function of leukocytes in the early phase of metastasis. Three specific objectives were pursued in this work:

1) Which subpopulation of leukocytes contributes to the metastatic process?

2) How do the leukocytes assist tumor cells during establishment of metastatic foci?

3) Do they affect their migration?

Since there is little known about the contribution of leukocytes to metastasis, this project was rather challenging for me.

Section II: RESULTS

Manuscript 1:
L-selectin dependent leukocyte facilitation of metastasis

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Running title: L-selectin facilitates metastasis

Key words: endothelial cells, extravasation, leukocytes, L-selectin, metastasis

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Abstract

Metastasis is supported by the formation of a tumor cell embolus, consisting of aggregated platelets and leukocytes adherent on tumor cells. While interactions between tumor cells and platelets and their contribution to metastasis have been studied, the participation of leukocytes in this mechanism remains to be elucidated. We previously showed that the absence of L-selectin on leukocytes leads to an attenuation of metastasis and to a reduced association between leukocytes and tumor cells. Here, we study the contribution of leukocytes to metastasis depending on L-selectin expression *in vitro* and *in vivo*. Granulocytes / monocytes were shown to help tumor cells to extravasate through the vascular endothelium in an L-selectin dependent manner. This process affects the endothelial cell layer and the vascular permeability. Depletion of monocytes led to an attenuation of metastasis, clearly indicating leukocytes as facilitators in metastasis. Taken together, leukocytes promote tumor progression during the early step of metastasis.

Introduction

Hematogenous metastases are the main cause of cancer-related death. To metastasize, tumor cells have to enter the bloodstream, evade innate immune surveillance and extravasate through the vascular endothelium. Tumor cell extravasation in carcinoma metastasis is known to be facilitated by the formation of platelet aggregates and leukocytes around tumor cells. These so called tumor cell emboli contribute to the establishment of metastatic foci (Honn et al., 1992b; Karpatkin and Pearlstein, 1981). The mechanism of platelet binding to tumor cells and its contribution to metastasis has been investigated. Coagulation and presence of platelets facilitated tumor cell metastatic spread in the pulmonary vasculature (Im et al., 2004). Platelets were shown to protect the tumor cells from the clearance by NK cells (Nieswandt et al., 1999) and to promote metastasis through P-selectin-mediated interactions with carcinoma cell-surface mucin ligands (Borsig et al., 2001a). Endothelial P-selectin expressed on the vasculature contributed also to the formation of hematogenous metastases (Ludwig et al., 2004). Vascular cell adhesion molecules P- and E-selectin were shown to contribute to metastasis by binding selectin ligands on tumor cells (Biancone et al., 1996; Garcia et al., 2007; Ludwig et al., 2004). Indeed in the absence of P-selectin, metastasis was attenuated (Borsig et al., 2001a). Leukocytes were associated with tumor cells on lung tissues but their function in the tumor cell emboli remained to be elucidated. Tumor microenvironment plays a major role in the contribution of leukocytes to tumor progression (Coussens and Werb, 2002; Fidler, 2002; Liotta and Kohn, 2001). Leukocytes have antitumor activity but increasing evidence indicates their potential role in supporting tumor progression (de Visser et al., 2006). Indeed, neutrophils were shown to enhance metastasis of tumor cells in several carcinomas (Queen et al., 2005; Tazawa et al., 2003; Welch et al., 1989). Interestingly, skin carcinogenesis was induced by inflammatory cells (Coussens et al., 2000). Clinical data show a correlation between high leukocytic infiltration into solid tumors and poor outcome of patients (Coussens and Werb, 2002).

L-selectin is constitutively expressed by granulocytes, monocytes, T and B cells and NK cells (Kansas, 1996) and mediates the rolling of leukocytes on endothelial cells as well as their binding to the endothelium during inflammation (Kansas, 1996; Ley, 2002; Sperandio et al., 2003). Interestingly, L-selectin ligands were found in the microenvironment of tumor cells being expressed on activated endothelium and on tumor cells MC-38 (Läubli et al., 2006). The absence of L-selectin attenuated metastasis in mice (Borsig et al., 2002), thereby directly

implicating leukocytes to this process. The effect of L-selectin was observed even in immunodeficient mice (T- and B- cell deficient), indicating that innate immune cells are most likely implicated in this metastatic process. Moreover, in the absence of L-selectin, the association of leukocytes with tumor cells was reduced. L-selectin promoted recruitment of leukocytes to tumor cells and their survival following their entrance in the vasculature (Laubli, Stevenson et al. 2006).

The present study was undertaken to identify the subtype of leukocytes implicated in the metastatic process and to define their role. We were able to show that both granulocytes and monocytes facilitate tumor cell transmigration in an L-selectin dependent manner.

Materials and methods

Syngeneic mouse/tumor model

Control wild-type (wt) C57BL6 mice were from The Jackson Laboratory (Bar Harbor, ME) and L-selectin-deficient mice (L-sel-/-) were backcrossed into the C57BL6 background as described previously (Borsig et al., 2002). Syngeneic MC-38 murine colon carcinoma cells (grade III) were derived from C57BL6 mouse strain (Borsig et al., 2002). Colon carcinoma cells were isolated as a mixed population of cells stably expressing green fluorescent protein (GFP) designated MC-38GFP (Borsig et al., 2002).

In vivo detection of leukocytes associated with tumor cells

Mice were intravenously injected with 4 x 10^5 MC-38GFP cells and lungs prepared at various time points as described previously (Borsig et al., 2001a). Frozen lung sections were stained with a monoclonal anti-CD11b antibody (Crockett-Torabi et al., 1995) from Becton Dickinson directly coupled with Alexa660 and anti-Ly6G-biotin antibody (Becton Dickinson), followed by Streptavidin-Alexa 546 (Molecular Probes). Finally, monoclonal antibody against BM8 was added (BMI, Basel), followed by goat-anti-rat-Alexa594 (Molecular Probes). Sections were analyzed on a Leica SP2 confocal laser scanning microscope. Serial section image stacks of a tissue section were acquired by Leica SP1 confocal laser scanning microscope and were analysed by Imaris® software (Bitplane AG, Zürich, Switzerland). At least ten images for every lung/time point were analyzed.

Isolation of lung microvascular endothelial cells

Mouse lung microvascular endothelial cells were isolated using a positive immunomagnetic selection (Wang et al., 2005). Briefly, 6 lungs were minced and digested with 0.1 % collagenase A (Gibco, Invitrogen) for 1 h at 37°C. Digested tissues were filtered through 100 µm and 40 µm sterile meshes (BD Biosciences). The cell suspension was incubated with rat anti-mouse CD31-FITC -0.4 µg per 1.10^6 cells- (BD Pharmingen^{TM}) and then incubated with 10 µl/10^7 cells of magnetic beads coated with anti-FITC isotype 1 (MACS Miltenyi Biotec). Cells bound to the beads were recovered with a magnetic separator (MS column, MACS Separation columns, Miltenyi Biotec) following manufacturer's instructions and resuspended in EC medium (DMEM low glucose containing 20 % FCS, 100 µg/ml of porcine heparin (Sigma), 100 µg/ml of endothelial cell growth supplement (BD Biosciences) and nonessential amino acids, HEPES, L-glutamine and antibiotics at standard concentrations). Cells were then

plated on 0.1 % gelatin (Sigma) coated six-well plates. Medium was changed every day the first 4 days in order to remove all contaminating non-adherent cells. At day 10, the cells were passaged on bigger plates and used for experiments at passages 2-5.

Isolation of granulocytes on a Percoll gradient

The isolation of granulocytes was performed on a three-layer Percoll gradient of 78 %, 69 % and 52 % Percoll (Boxio et al., 2004). After centrifugation, granulocytes were collected at the 78-69 % interface followed by a lysis of contaminating red blood cells with PharMlyse (BD Pharmingen). The purity of the obtained granulocytes was always above 95 % among leukocytes.

Granulocytes and monocytes derived from clones of immortalized bone marrow cells

Granulocytes and monocytes from wt or L-sel-/- mice were obtained in high number by conditional derivation of primary murine marrow progenitors (Sykes and Kamps, 2001).
Briefly, bone marrow from the femur and tibia of wt and L-sel-/- mice were isolated after ammonium sulfate lysis of erythrocytes and centrifugation onto a Ficoll-Paque gradient (Ficoll-Paque PLUS, Amersham). Ficoll-purified progenitors were then pre-stimulated 48h in 25 ng/ml murine SCF, 10 ng/ml murine IL-3 and 20 ng/ml murine IL-6 (cytokines from Cell sciences) for an optimized retroviral transfection. The marrow progenitors were retrovirally infected by spinoculation in the presence of lipofectamine (Invitrogen). The retroviral vector expressed the fusion oncoprotein E2a/Pbx1 fused to the hormone binding domain (HBD) of the estrogen receptor for its conditional expression. The infected progenitors were cultured in RPMI 1640 from Gibco with 10 % FCS, 1 % GM-CSF-conditioned medium from B16 melanoma expressing the *Csf2* cDNA and 1 μM estrogen from Sigma. Immortalized myeloid progenitors were selected by removal of nonadherent progenitor cells every 3 days to a new well during 3 weeks. Differentiation of progenitors committed exclusively to granulocytes or monocytes required cloning performed using 96-well microtiter plates. Clones were identified by screening for Gr-1 expression after retrieval of estradiol from the culture medium.

Preparation of tumor-conditioned medium (TCM)

Tumor cells MC-38 (100’000/ml) were added to endothelial cells grown till confluency in DMEM 5 % FCS for 24 hours. TCM was harvested, centrifuged and stored at -20°C until use.

Assessment of tumor cell transmigration

Mouse lung microvascular endothelial cells were grown in 0.3 ml EC medium ($1.7x10^5$ cells/ml) in the upper chamber of a 6.5 mm diameter insert (8 µm pore size Falcon TM cell culture inserts, Becton Dickinson Labware), with 0.8 ml of EC medium in the lower chamber at 37°C for 24 h until a confluent monolayer was reached. For leukocyte-assisted tumor cell MC-38GFP migration assay, granulocytes and monocytes derived from clones of immortalized bone marrow cells or granulocytes isolated on a Percoll gradient were used. Granulocytes (200'000), monocytes (40'000) were mixed to tumor cells MC-38GFP (20'000) in a total volume of 0.3 ml DMEM with 5 %FCS and added to the upper chamber above the endothelial cell monolayer. 0.5 ml of TCM was added to the lower chamber. After 16 hours of co-culture, inserts were scraped to remove non migrated tumor cells, fixed and stained with Dapi. Migrated tumor cells were counted on 40 view fields under the fluorescence microscope (Zeiss) at 40 x magnification. Migrated leukocytes were collected in the lower chamber and counted. Statistical significance was determined by the Student's *t* test.

Cytoskeletal remodeling

To demonstrate that pulmonary endothelial cells are capable of responding to the presence of tumor cells, granulocytes and monocytes-mediated activation, we investigated the distribution of F-actin as described previously (Zarbock et al., 2006). Briefly, 20'000 endothelial cells were allowed to grow to confluency on gelatin-coated glass coverslips for 48 hours. 20'000 tumor cells in the presence of granulocytes (200'000) and monocytes (40'000) were added in DMEM with 5 % FCS or in TCM. Endothelial cells alone served as control. After 4 h of incubation at 37°C, the cells were fixed (PFA 3 %), permeabilized (0.1 % saponin, Sigma-Aldrich) and stained with FITC-phalloidin (40µg/ml, Invitrogen Corp.). Coverslips were mounted on glass slides. The analysis was performed using a Leica SP5 confocal laser scanning microscope and Imaris® software (Bitplane AG, Zürich, Switzerland). Cell areas and intensities were determined with ImageJ program (EMBL).

Pulmonary microvascular permeability

Determination of pulmonary microvascular permeability in wt and L-sel-/- mice was performed using the Evans Blue dye extravasation technique (Reutershan et al., 2006). Briefly, 400'000 MC-38 tumor cells were injected intravenously in mice for 24 hours. 2 mg of Evans Blue (Sigma-Aldrich) was injected intravenously 30 minutes prior to euthanasia. Lungs were perfused through the spontaneously beating right ventricule to remove intravascular dye. Lungs were removed and homogenized. Evans Blue was extracted by incubation with 2 volumes formamide at 60°C for 18 hours. After centrifugation, the optical density of the supernatant was measured by 620nm. Extravasated Evans Blue in micrograms was determined in the different animal groups.

Clodronate depletion of monocytes and experimental metastasis assay

Mice were intravenously injected with 2 mg of clodronate loaded liposomes as described previously (Zeisberger et al.). After 48 h mice were intravenously injected with

3.5×10^5 MC-38GFP cells. After 28 days, mice were sacrificed and metastasis was evaluated by counting of lung foci and/or detecting of GFP fluorescence in lung homogenate as previously described (Borsig et al., 2002).

Results

L-selectin mediates association of innate immune cells to metastatic foci

Attenuation of metastasis observed in L-selectin deficient mice identified leukocytes in this process. Tumor cells were shown to be associated with leukocytes in an L-selectin dependent manner (Läubli et al., 2006). To determine the nature of the immune cells in the vicinity of tumor cells in the lungs, we analyzed tumor associated leukocytes in lung tissues. For this, lungs were fixed at different time points following injection of tumor cells. Lung sections were stained with antibodies recognizing different populations of leukocytes. Monocytes were detected for their positive Mac-1 staining, granulocytes for Gr-1 and Mac-1 and macrophages for BM8. Tumor cells were associated with monocytes, macrophages and granulocytes and the contact of these immune cells with tumor cells was reduced in lungs of mice deficient for L-selectin 12 till 36 hours after tumor injection (Figure 1). These results suggest that L-selectin on granulocytes / monocytes / macrophages has an important role in the metastatic spread of tumor cells.

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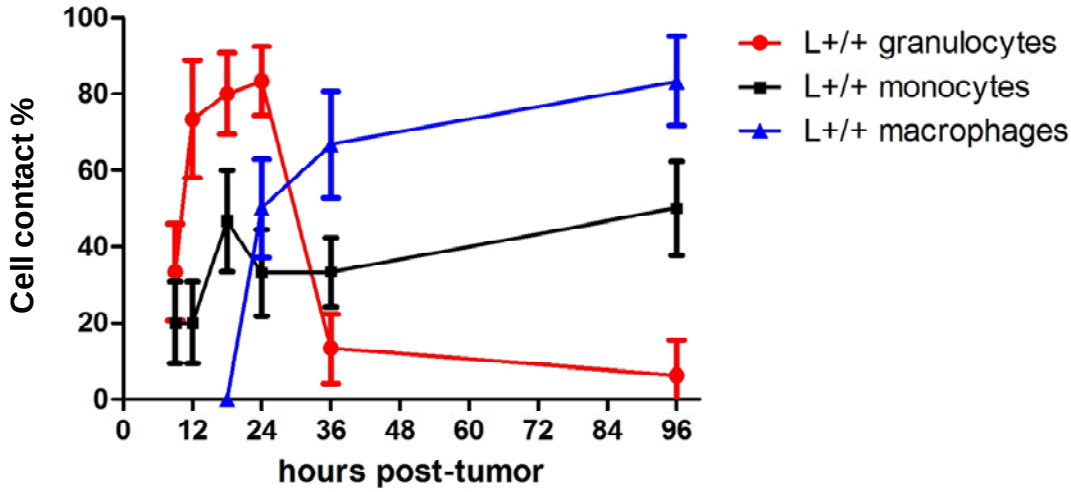

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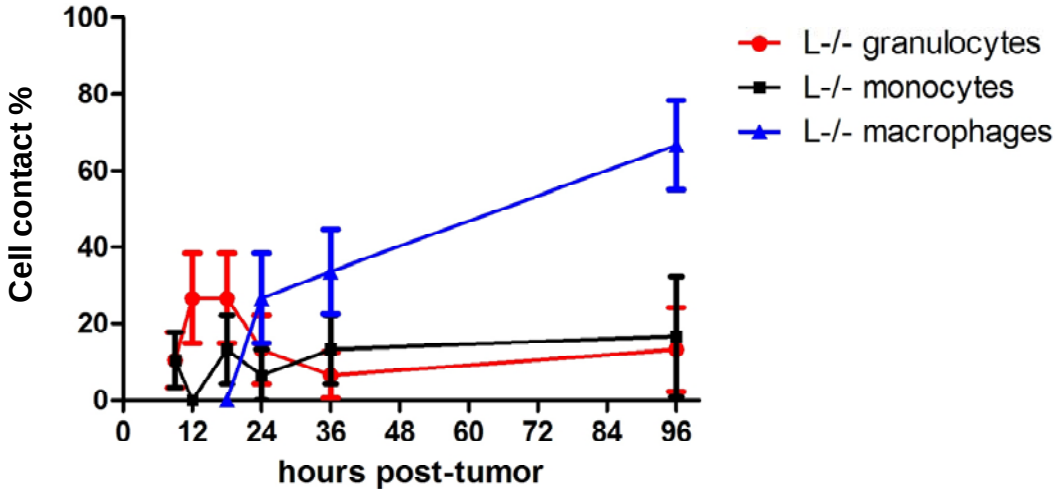


Figure 1: *Cell-cell contact between tumor cells and immune cells is reduced in mice deficient for L-selectin.* The figures show the percentage of contact between tumor cells and immune cells on lung sections coming from wt (A) and Lsel-/- mice (B) at different time points following tumor cell injection. $P<0.05$ (unpaired Student's *t* test). Results shown were taken from the thesis of Peter Jäggi and Heinz Läubli.

Leukocytes enhance transendothelial migration of tumor cells in an L-selectin dependent manner

Monocytes and granulocytes have been shown to be associated with tumor cell emboli in an L-selectin dependent manner in lung sections *in situ* (Figure 1). In order to investigate the role of L-selectin and the function of leukocytes associated with tumor cells, we proceeded to a transmigration assay *in vitro*. It was of interest to determine whether granulocytes (G) or monocytes (M) or both affect the migratory capacities of tumor cells through the endothelium.

For this, tumor cells were added to an endothelial cell monolayer in the presence of granulocytes and/or monocytes and their migratory capacities were determined after 16h of co-culture (Figure 2).

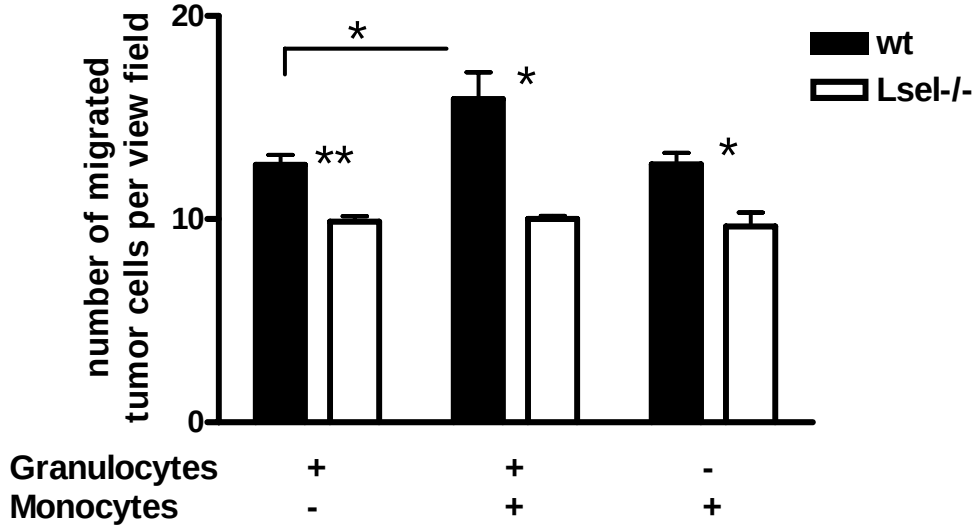


Figure 2: *Granulocytes and monocytes enhance the transmigration of tumor cells through endothelial cells (EC) in an L-selectin dependent manner.* Cells were mixed at the following ratios: for one part of tumor cell (20'000), ten parts of granulocytes (200'000) and two parts of monocytes (40'000) were added. Migration of tumor cells alone was normalized at 10 cells/view field. The figure is a summary of at least three independent experiments. * P<0.05, ** P<0.001 (unpaired Student's *t* test). *Columns*, mean; *bars*, SEM.

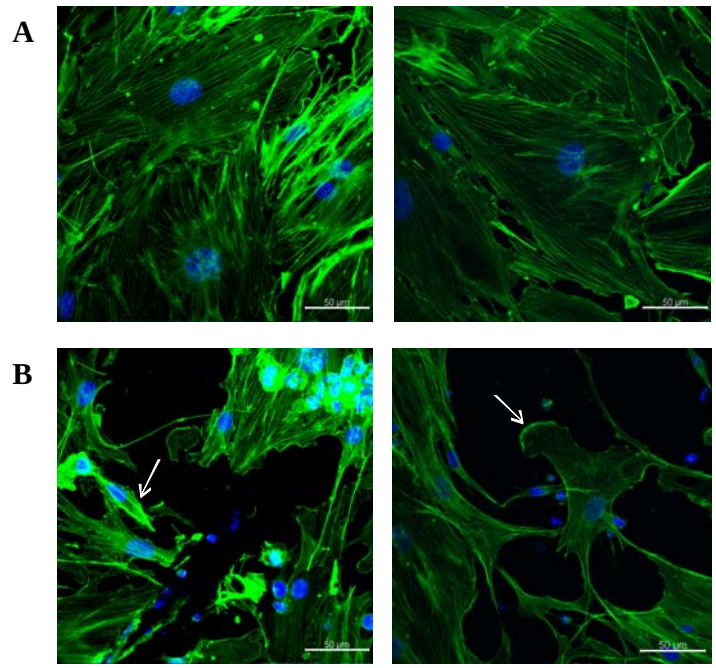
Tumor cell migration was facilitated by both classes of leukocytes, granulocytes and monocytes, in an L-selectin dependent manner (Figure 2). We observed a 30 % increase of tumor cell migratory capacities by the presence of granulocytes. Interestingly, this positive effect in the presence of leukocytes was amplified by monocytes and granulocytes added together with tumor cells. In this case, an increase of 60 % compared to basal migratory capacities of tumor cells was observed. Innate immune cells derived from L-selectin deficient mice were not able to potentiate tumor cell migration, regardless of the mixture. Thus, L-selectin on leukocytes is implicated in the promotion of tumor cell migration through endothelium.

In order to perform this assay, we used monocytes and granulocytes cloned and differentiated from bone-marrow progenitor cells (Sykes and Kamps, 2001) because it was not possible to isolate mouse monocytes in a high number. We performed the same assay using blood granulocytes isolated on a Percoll gradient and tumor cell transmigration was affected in the

same way independently of the use of blood granulocytes or granulocytes derived from clones. Thus granulocytic and monocytic clones of immortalized bone marrow cells possess the same functions as native leukocytes. The ratio of monocytes to tumor cells was crucial for their migration. Indeed, when five parts of monocytes for one part of tumor cells were used, the positive effect on migration was abolished (Supplementary Figure 1). Thus, monocytes seem to have a pivotal role in the transmigration process. Interestingly, tumor cell migration was in correlation with the number of migrating leukocytes. Indeed, we observed that granulocytes derived from wt mice in interaction with tumor cells and endothelial cells are functionally more active and migrated in a higher proportion through endothelial cells as compared to granulocytes derived from L-selectin deficient mice (Supplementary Figure 2). This suggested that leukocytes could be activated in an L-selectin dependent manner and possibly enhanced tumor cell extravasation. Moreover, tumor cell conditioned medium (TCM), prepared from incubation of tumor cells with endothelial cells, and used at the bottom well of our assay may contain chemotactic agents important for tumor cell migration.
These findings show that L-selectin mediated interactions of leukocytes at the site of extravasation contribute to transendothelial migration of tumor cells.

L-selectin dependent tumor cell transmigration affects endothelium

Leukocytes enhanced tumor cell migration through endothelial cells. To investigate whether the endothelial cell monolayer was affected by the presence of tumor cells and leukocytes, we proceeded to a co-culture assay. Tumor cells and leukocytes were added to endothelial cells (EC) grown on confluency on coverslip for 4 hours and the EC response was analyzed by staining for phalloidin binding to actin filaments (Figure 3 A and B).



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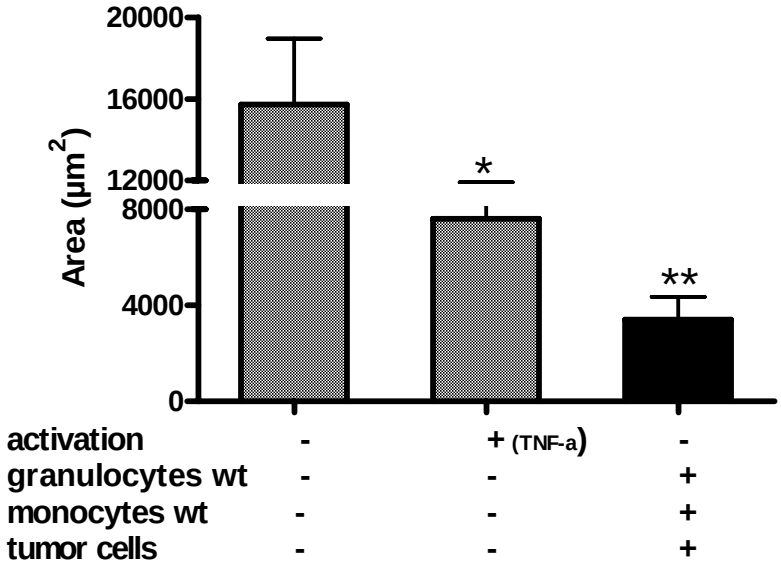

Figure 3: *Endothelial cell response to addition of tumor cells, granulocytes and monocytes was investigated by F-actin localization.* F-actin was localized by phalloidin staining (green). **A** In case of endothelial cells alone, the monolayer stayed intact. **B** Tumor cells, granulocytes, monocytes activated the endothelial cells at various sites. Endothelial cells appear in a polygonal retracted shape (arrow in the left panel). Images are representative of three independent experiments with similar results. **C** Area of the retracted EC in comparison with normal EC in an intact monolayer defined as described in Materials and Methods. * $P<0.05$, ** $P<0.005$. *Columns*, mean; *bars*, SEM.

In the presence of tumor cells and leukocytes EC layer retracted at many places and a general separation of adjacent cells was observed in many locations (Figure 3B, left panel). Endothelial cells appeared in a polygonal retracted shape with increasing intensities of staining for actin bundles at the cell border (Figure 3B, arrows in left and right panels). This EC retraction follows a reorganization of the actin cytoskeleton and a formation of stress fibers (Zarbock et al., 2006). At these sites of EC barrier interruptions, the cells were significantly smaller (Figure 3C). The retraction of the cells was not dependent on L-selectin expression of leukocytes. The presence of TCM also led to a retraction of the endothelial cell layer. This result shows that tumor cells in the presence of leukocytes may be able to activate endothelial cells by provoking their retraction.

To further investigate this finding *in vivo*, the pulmonary vascular permeability was tested after injection of tumor cells. Evans Blue was injected 30 minutes preceding the perfusion of the lungs. The amount of Evans Blue in lung homogenates was then quantified as described in the Materials and Methods section (Figure 4).

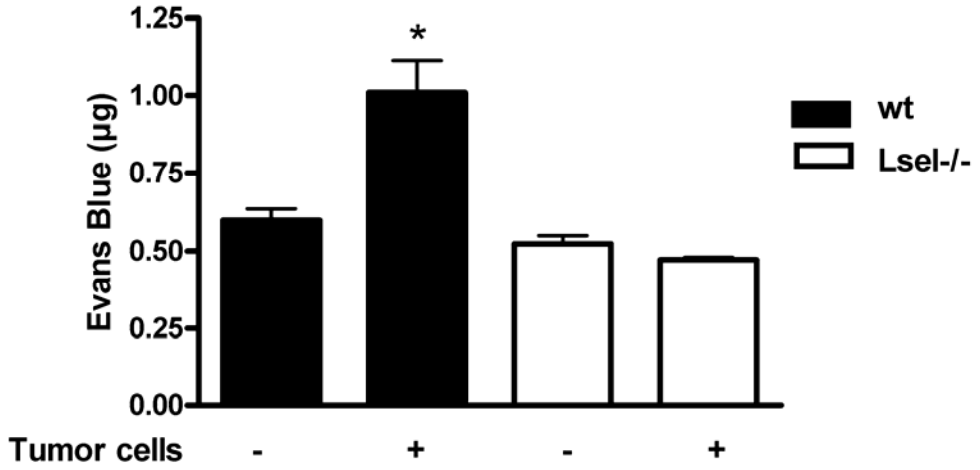

Figure 4: *Pulmonary microvascular permeability is enhanced in an L-selectin dependent manner.* The figure shows the permeability of the lungs 24 h following tumor cell injection. * P<0.05 (unpaired Student's *t* test), n = 4. *Columns*, mean; *bars*, SEM

The vascular leak of the lungs after the injection of tumor cells was enhanced by 65 % in the wt mice. There was no enhancement observed in absence of L-selectin. Thus L-selectin presence on leukocytes correlates with an enhancement of the vascular permeability.

Leukocytes and tumor cells are able to induce a physiological change of the endothelial barrier, most probably at the extravasation site of tumor cells.

These data show that tumor cells and leukocytes alter the EC layer *in vitro*. Confirming this observation, L-selectin on leukocytes increased the vascular permeability after tumor cell injection mice. These findings suggest that during the process of tumor cell extravasation endothelial cells are responsive to the presence of leukocytes and tumor cells thereby modulating their physiological properties in an L-selectin dependent manner.

In vivo depletion of monocytes leads to a reduction of metastasis

The finding that L-selectin on leukocytes in the presence of tumor cells modulates the endothelial cell layer response led us to suggest that leukocytes contribute also to extravasation of tumor cells through the endothelial barrier *in vivo*. Previously, we observed that the depletion of L-selectin *in vivo* by injection of a functionally blocking antibody 6-12 hours after tumor cell injection led to an attenuation of metastasis (Läubli et al., 2006). When granulocytes were depleted by injection of Ly6G Ab in mouse (Sitia et al., 2002), no attenuation of metastasis was observed. Depletion of granulocytes did not result in an attenuation of metastasis, most probably due to the fact that it lasted 5 days in the circulation. Indeed, granulocytes were not in the circulation within the time frame of the initial clearance of tumor cells (Läubli et al., 2006). More work is needed to optimize the depletion of granulocytes in order to delineate their action *in vivo*. To test whether monocytes contributed to metastasis, mice were intravenously injected with clodronate encapsulated in liposomes. Forty-eight hours following monocyte depletion, tumor cells were intravenously injected. After 4 weeks, metastasis was highly attenuated in monocyte-depleted wt mice as shown by quantification of the metastatic foci (Figure 5-A and B). The level of metastasis was even lower than the one observed in L-selectin deficient mice. Thus circulating monocytes promote metastasis *in vivo*. The function of monocytes in metastasis was confirmed by evaluating the presence of monocytes in the vicinity of tumor cells by immunofluorescence staining of lung sections of these depleted mice (Figure 5-C). Monocytes ($F4/80^{+}$-$Mac\text{-}1^{+}$) were in close proximity of tumor cells in non depleted lungs (Figure 5-C, left panel). In clodronate-depleted lungs, the association of monocytes with tumor cells was highly reduced (Figure 5-C, right panel). Moreover, on lung sections of these depleted mice, total number of monocytes was highly reduced and the total number of tumor cells was decreased by factor 3 (data not shown). Alveolar macrophages stayed untouched in both wt and depleted mice (data not

shown). Taken together, these results strongly indicate a major role of monocytes in the formation of metastases.

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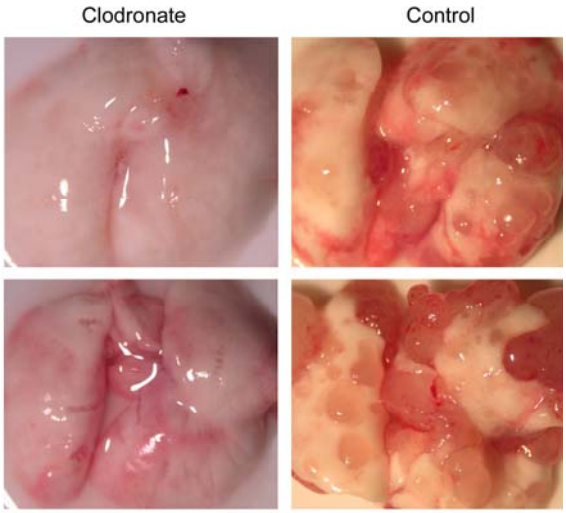

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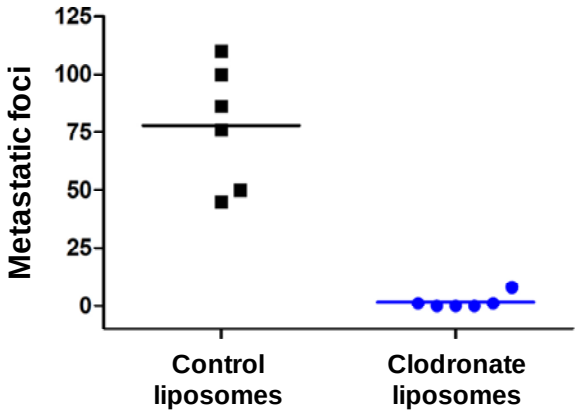

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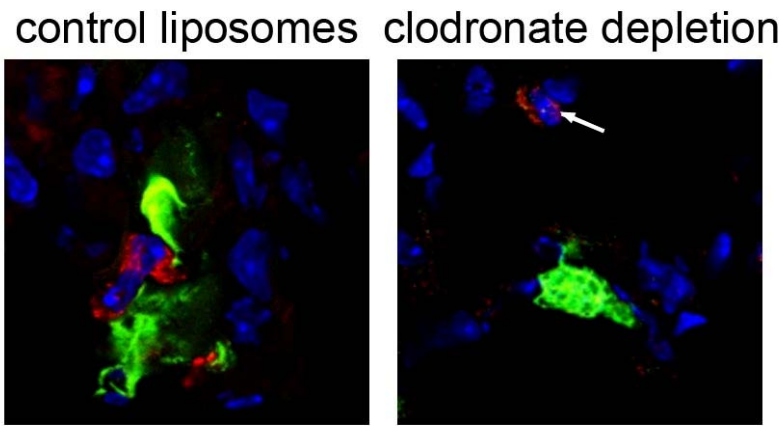


Figure 5: *Depletion of monocytes in vivo attenuates metastasis.* **A,** wt and monocytes depleted mice were injected with MC-38GFP cells and mice were euthanized after 26 days and dissected lungs were evaluated for metastatic foci. **B,** The extent of metastasis was analysed by GFP fluorescence read out in lung homogenates. $P<0.001$ (unpaired Student's *t* test). **C,** Mice depleted of monocytes by clodronate liposome injection were 48 h later injected with 3.5 x 10^5 MC-38GFP cells and after another 24 h sacrificed. Representative images of lung sections stained with anti-CD11b antibody (red) and GFP tumor cells (green) and nuclei stained with DAPI (blue) are presented. Injection of clodronate liposomes led to reduced monocyte association with tumor cells (right panel), while in controls (empty liposomes) association with tumor cells could readily be seen (left panel). Note, that monocytes/macrophages could be detected in the depleted lungs, but are not tumor associated (arrow in the right panel).

Discussion

Hematogenous metastasis is facilitated by tumor cell-platelet-leukocyte embolus formation. Previous studies showing an enhancement of selectin ligands on cancer cells associated with tumor progression suggested an involvement of selectins in cancer development (Hakomori, 1996; Kannagi et al., 2004; Kim and Varki, 1997). Endothelial E- and P-selectin were shown to promote metastasis in several mouse models (Biancone et al., 1996; Borsig et al., 2002; Kannagi et al., 2004). Platelet-tumor cell interactions was shown to be primarily mediated by P-selectin, while the inhibition of these interactions was associated with reduction of metastasis (Borsig et al., 2001a; Borsig et al., 2002; Garcia et al., 2007). Our previous findings, that L-selectin deficiency is associated with attenuation of metastasis, clearly implicated leukocytes in this process (Borsig et al., 2002). L-selectin promoted tumor cell survival, most probably by action of leukocytes found to be associated with tumor cells (Läubli et al., 2006). Here we provide the first evidence that cell-cell contacts among tumor cells, granulocytes / monocytes are L-selectin dependent and are associated with leukocyte recruitment to metastasizing tumor cells in the lung (Figure 1). The association between immune cells and tumor cells has been shown to correlate with metastasis (Coussens et al., 2000; Lin et al., 2001; Queen et al., 2005; Tazawa et al., 2003). Neutrophil infiltration of tumors led to the acquisition of a metastatic phenotype of otherwise benign murine fibrosarcoma cells (Tazawa et al., 2003). Moreover, tumor associated monocytes have been shown to directly participate in tumor progression (Lin et al., 2001). Macrophages recruited at the site of primary tumor are able to increase the metastatic potential of tumor cells. Interestingly, bone marrow-derived cells can lead to skin carcinogenesis (Coussens et al., 2000). Macrophages recruited to the site of primary tumor can increase the metastatic properties of tumor cells. Their presence close to tumor cells seems to be determinant for the further outcome of tumor cells. Little is known on the role of circulating monocytes in metastasis. Interestingly circulating monocytes were associated with tumor cells suggesting that they could participate to the formation of metastases in our mouse model.

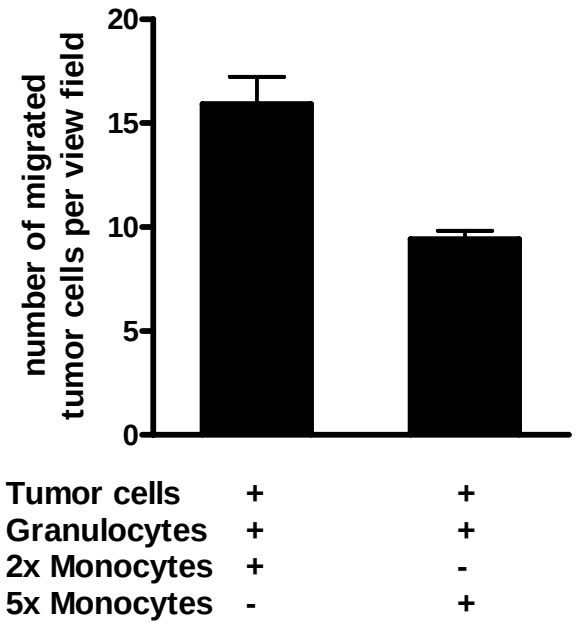
To elucidate the function of the associated leukocytes, we first focused on the role of granulocytes and monocytes in a transmigration assay. We suggest that monocytes and/or granulocytes could assist tumor cells to breach the endothelial barrier. We could show that granulocytes / monocytes promote tumor cell transmigration through endothelium (Figure 2). The link between inflammation, presence of granulocytes and the enhancement of tumor migration has already been suggested (Welch et al., 1989). For instance, neutrophils were

enhancing the metastatic potential of rat adenocarcinoma cells, by facilitating invasion into basement membrane (Welch et al., 1989). We showed that granulocytes and monocytes promoted tumor cell migration through endothelium in an L-selectin dependent manner (Figure 2) suggesting that L-selectin affects metastasis at the site of tumor cell extravasation. The observed tumor cell migration differences could explain the attenuation of metastasis observed in mice deficient for L-selectin and could implicate direct cell-cell contact between tumor cells, innate immune cells and endothelium. We made then the interesting observation that this enhanced tumor cell transmigration was due to a physiological change of underlying endothelial cells. Endothelial cells that line the vasculature have close cell-cell association and their permeability is regulated by many growth factors and cytokines (Stockton et al., 2004). We observed a retraction of the endothelial layer characterized by altered cell-cell contacts (Figure 3A and B) and a high dependence on cell-cell contacts between leukocytes-tumor cells and endothelial cells. The enhancement in vascular permeability was L-selectin dependent (Figure 4). Thus, endothelial cells seem to be a key player in the facilitation of metastasis. Tumor cells have been shown to cause endothelial cell retraction (Honn et al., 1994; Lee et al., 2003). In agreement with our findings, other groups described a loss of endothelial cell integrity during tumor cell transmigration (Heyder et al., 2002; Weis et al., 2004). Indeed, the production and release of VEGF by tumor cells led to a breakdown of endothelial barrier function through a disruption of adherent junctions. This, in turn, caused rapid tumor cell extravasation and metastasis (Weis et al., 2004). Another study showed that the vascular endothelium was irreversibly damaged at the site of tumor cell extravasation by using real-time visualization of tumor cell/endothelial cell interactions during transmigration. The damaged endothelium was undergoing apoptosis (Heyder et al., 2002). In our assay, TCM also had an effect on endothelial cell activation (Figure 3B). We suggest that TCM contains chemokines -being secreted by tumor or endothelial cells or both- that modulate the endothelial cell layer. TCM, leukocytes and tumor cells are pivotal in EC layer activation. When only one component is absent, the endothelial cell layer is less affected. Both cell-cell contact and cytokine secretion may be relevant also for the transmigration *in vivo*.

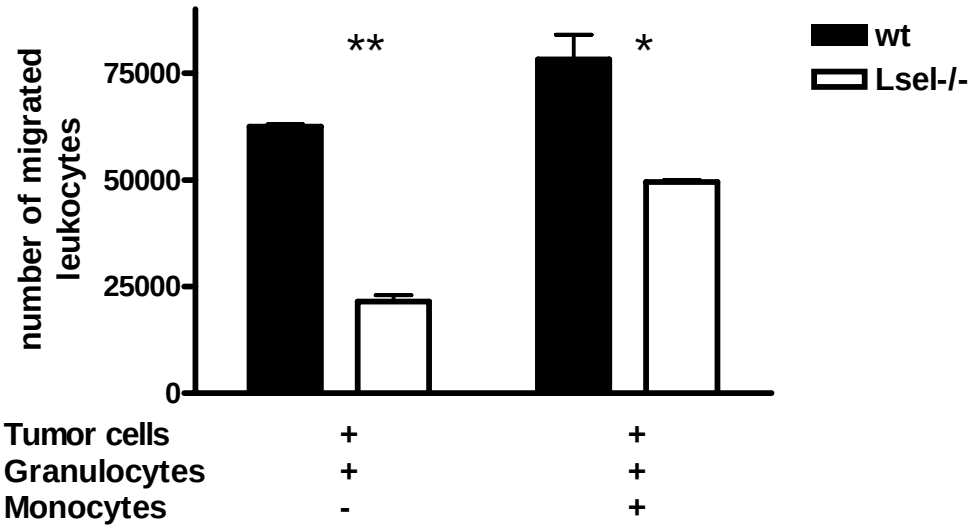
Moreover, the direct role of monocytes was confirmed *in vivo*. The depletion of circulating monocytes led to attenuation of metastasis (Figure 5), documenting their essential contribution to the promotion of metastasis. Recruited circulating monocytes may facilitate tumor progression by increasing the migratory capacities of tumor cells and by acting on the permeability of the endothelial cells.

In conclusion, our study proposes recruited circulating monocytes as pivotal partners of tumor cell metastasis and L-selectin as a key contact molecule between leukocytes, tumor cells and endothelial cells for the establishment of metastasis.

Supplementary Figures



Supplementary Figure 1: *Migration of tumor cells in the presence of granulocytes and monocytes at different ratios.* The figure presents the transmigration of tumor cells through EC in the presence of granulocytes and monocytes. Five parts (5x) or two parts (2x) of monocytes were added for one part of tumor cells. Migration of tumor cells was normalized at 10 cells/view field for the migration of tumor cells alone. This figure is representative of six independent experiments. $p<0.005$ (unpaired Student's *t* test). *Columns*, mean; *bars*, SEM.



Supplementary Figure 2: *Migration of leukocytes accompanying tumor cell transmigration.* The figure presents the transmigration of leukocytes through EC in the presence of tumor cells.

** P<0.005, * P<0.05 (unpaired Student's *t* test). *Columns,* mean; *bars*, SEM.

Additional data

Endothelial cell isolation

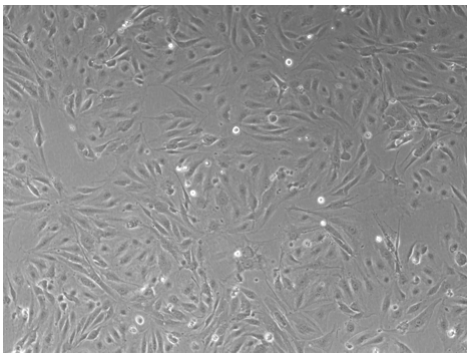
We studied experimental metastasis in a mouse model, where tumor cells metastasize primarily to the lungs. Therefore, to study the role of leukocytes in initiation of metastasis, endothelial cells were isolated from mouse lung tissue.

Endothelial cells were isolated according to the Methods previously described in Manuscript1 (Wang et al., 2005). The isolation and long-term culture of primary murine lung endothelial cells was difficult due to overgrowth of contaminating cells, in particular fibroblasts. Here is a brief summary of the optimized protocol for the isolation of primary lung endothelial cells:

- we were not proceeding to HistoDenz (containing metrizamide) step of EC enrichment by lymphocytes retrieval as we did not find any advantage to this step (Fields et al., 1998). Indeed, the co-purified lymphocytes, binding also CD31, were found in the supernatant of our culture (tested by CD5 expression) and easily retrieved by aspiration and washed from culture medium,
- we were doubling the concentration of primary antibody CD31-FITC and reducing the concentration of the beads (Tiruppathi et al., 2002),
- we always tried to obtain a single-cell suspension before addition of primary antibody in order to avoid the co-purification of unwanted cells,
- we cultured EC on gelatin, not on matrigel,
- we were not using any treatment containing trypsin-EDTA solutions, because the murine CD31 epitope could be damaged by this enzymatic treatment (Marelli-Berg et al., 2000).

Following isolation, EC grew in several clusters that formed a monolayer within 10 days and showed the characteristic cobblestone morphology (Figure 1A). Expression of CD31 surface molecule, an EC marker, was performed by the help of flow cytometry. It indicated the purity of the isolated EC (Figure 1B).
Finally from 6 lungs, $20x10^6$ pure endothelial cells were obtained (about 15 days after the isolation, 3rd passage).

A **B**



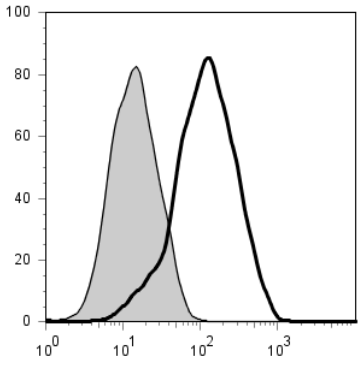


Figure 1: *Isolated mouse endothelial cells.* **(A)** This picture shows the typical cobblestone morphology of the isolated endothelial cells. (B) Endothelial cells were stained with CD31-FITC and analyzed by flow cytometry. CD31 staining is represented by a bold solid line. Filled area represents control unstained cells. EC population was 100 % pure.

Granulocyte and monocyte isolation

In order to use granulocytes and monocytes in our co-culture experiments, we needed to isolate them in high number and in a non activated state.

Method 1:

Neutrophils were first isolated from the peripheral blood mononuclear cells by using a cocktail of antibodies and a magnetic column as described by Cotter and his colleagues (Cotter et al., 2001). Briefly, mouse blood was collected by cardiac puncture and platelets were removed after dextran sedimentation of erythrocytes (1% dextran in PBS, 10' at 37°C). Erythrocytes were lysed by ammonium chloride solution (PharmLyse, BD Biosciences, San Diego, CA). Blood mononuclear cells were then incubated with a cocktail of antibodies against specific markers for unwanted cells, lymphocytes and monocytes, followed by incubation with secondary antibody coated magnetic beads. The antibodies used were CD45R (B220) for B-lymphocytes, CD5 for T and B lymphocytes, CD2 for T and B lymphocytes and NK cells, F4/80 for mature monocytes and ICAM-1 for monocytes. Neutrophil-rich effluent was then collected, stained with the marker Gr-1 and analyzed by flow cytometry.

The depletion of monocytes had to be optimized and finally, a pure population of neutrophils with a yield of 200'000 neutrophils / ml blood was obtained. Unfortunately, the obtained neutrophils were activated. A characteristic loss of size and granularity by analyzing the cells before and after isolation by flow cytometry was observed. In the presence of PMA, a known activator of granulocytes, isolated cells were even not able to shed L-selectin anymore (Crockett-Torabi et al., 1995). The paper published by Cotter and his colleagues claimed that isolated neutrophils were still L-sel +/+, a phenomenon that we were never able to reproduce even after optimizing and controlling each step for the presence of potential activators. This activation could occur during the negative selection.

Method 2:

Another method was used by isolating granulocytes on a three-layer Percoll gradient of 78 %-69 % and 52 % Percoll, as described previously in the Methods of Manuscript 1 (Boxio et al., 2004). For this isolation, whole blood was loaded on the 3 step-gradient without using dextran sedimentation of RBC for the platelet removal. EDTA 15 mM was used since blood clotting is leading to platelet activation and potential binding to leukocytes. Neutrophils were

collected at the 78-66 % interface (Figure 2) in tubes coated with 1 % BSA in order to avoid their loss on the walls (Bedner et al., 1997). The obtained neutrophils were not activated and >95 % pure.

The limitation of this method was the low yield of 150-350'000 neutrophils/ml blood. Still, these granulocytes were used to verify the results obtained with granulocytes derived from immortalized bone marrow cells.

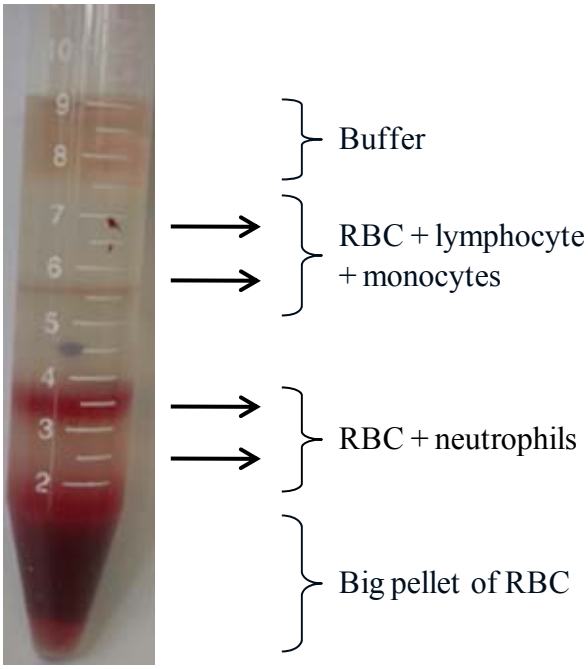


Figure 2: *Isolation of neutrophils on a three-step Percoll gradient.*

Method 3:

We finally found a way to obtain a high number of granulocytes and monocytes by culturing and cloning immortalized mouse bone marrow cells (Sykes and Kamps, 2001) as described previously in the Methods of Manuscript 1. The yield of granulocytes isolated on a Percoll gradient of 150-350'000 granulocytes/ml blood was indeed too low to permit us to use these cells for all settings and experiments. Moreover, the cloning procedure gave the advantage to obtain also monocytes in a high number.

Briefly, the cell lines were established by conditional immortalization of primary murine marrow progenitors. For this, the construct E2a/Pbx1, a fusion protein that blocks myeloid differentiation in mouse marrow in culture was subcloned into the murine stem cell virus (MSCV) retroviral vector used for immortalization. The expression of this fusion protein was under the control of the hormone binding domain of the estrogen receptor. Uninfected

primary mouse marrow cells differentiate into short-lived granulocytes and monocytes. Expression of E2a/Pbx1 immortalizes GM-CSF-dependent myeloid progenitors in the presence of estradiol in the culture medium. In the absence of estradiol, the cells differentiate in granulocytes (80 %) and monocytes (20 %).

After a cloning procedure, granulocytic and monocytic progenitors were selected for both wt and L-sel-/- mice. Differentiated granulocytes and monocytes were tested for Gr-1 expression, a granulocytic marker (Figure 3 A and B) and F4/80 expression, a monocytic marker (Figure 3 C and D).

Interestingly, monocytes coming from Lsel-/- mice were difficult to obtain. The percentage of cells differentiating in monocytes was clearly below 20 % and they were growing more slowly than wt monocytes. Moreover, we were not able to keep them for more than 20 passages in culture.

To control whether granulocytes were in a non activated state, 10^{-7}M of PMA was added, a known activator of granulocytes, for 10 min at 37°C. The activation of granulocytes was expressed as a function of L-selectin shedding and Mac-1 up-regulation, both markers for granulocytes activation (Crockett-Torabi et al., 1995). The obtained cells were in a non activated state (figure 3 E and F).

Conclusion:

Studying the role of murine innate immune cells in inflammation processes is expensive and time-consuming. Indeed, monocytes and granulocytes are non-mitotic cells and they need to be derived from large numbers of mice (Methods 1 and 2) so that their role can be studied in wt and Lsel-/- mouse models. By the help of the Method 3, unlimited quantities of functional monocytes and granulocytes from wt and Lsel-/- mice were produced.

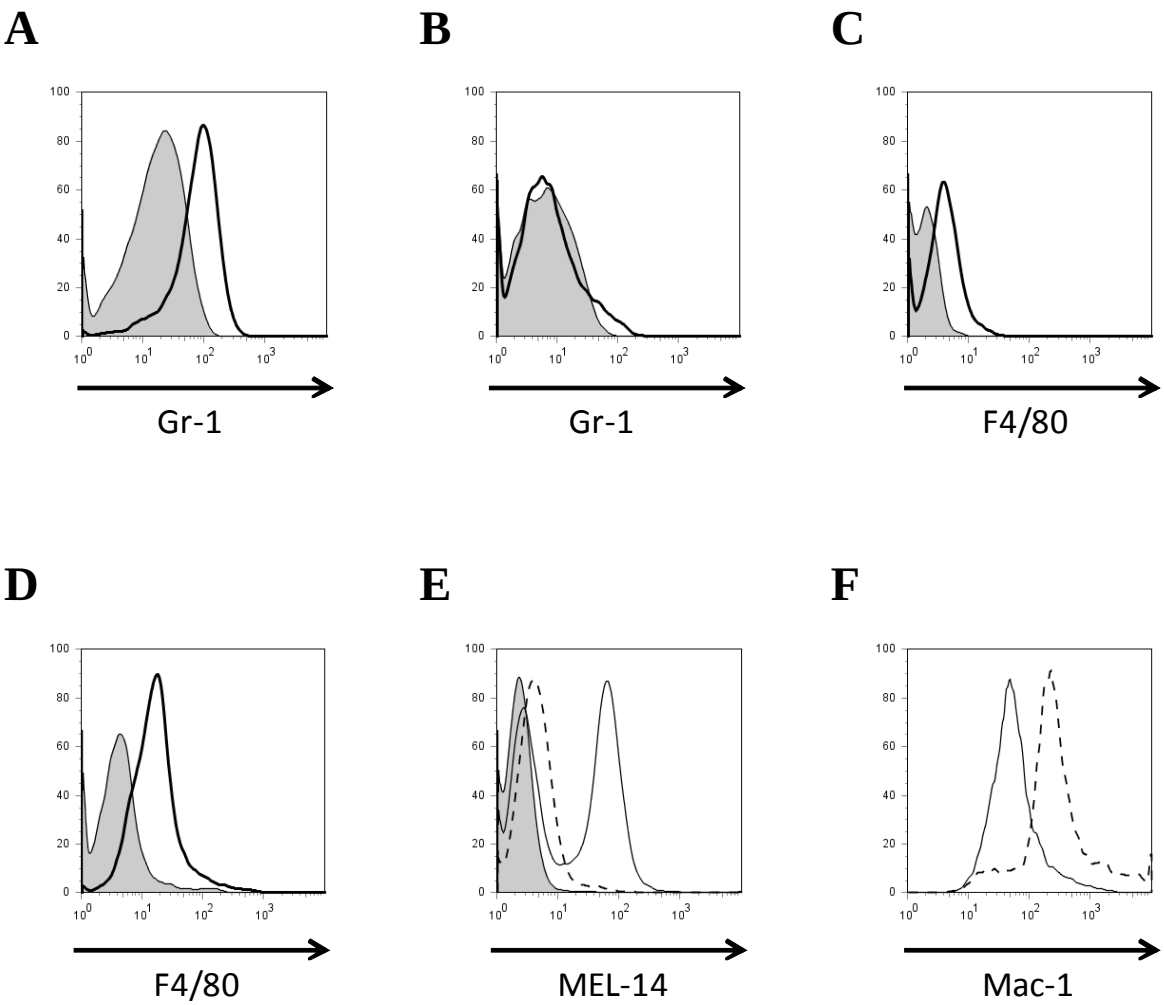


Figure 3: *Characterization of granulocytes and monocytes derived from clones of immortalized bone marrow cells.* Granulocytes **(A)** and monocytes **(B)** were stained with Gr-1 and analysed by flow cytometry. Filled area represents the Gr-1 staining of progenitor cells.

Granulocytes **(C)** and monocytes **(D)** were stained with F4/80. Filled area represents granulocytes **(C)** or monocytes **(D)** stained with the secondary Ab only.

Granulocytes were stained with MEL-14 **(E)** and Mac-1 **(F)** before and after activation by PMA. Thin lines represent the staining before activation while dashed lines represent the staining after PMA activation. Filled area represents the staining with a secondary Ab.

Activation of granulocytes by tumor cells

The direct activation of granulocytes in the presence of tumor cells was studied since leukocytes were playing a central role in tumor cell transmigration (see Manuscript 1). To test this, tumor cells and granulocytes were incubated at different ratios (one part of tumor cells for one part or eight parts of granulocytes) and incubated for 4 hours at 37°C.

Activation of granulocytes is characterized by an up-regulation of the Mac-1 cell surface marker and by the shedding of L-selectin molecule (Crockett-Torabi et al., 1995). Surprisingly, granulocytes were not activated after this incubation in the presence of tumor cells. Indeed, they did not over-express Mac-1 and granulocytes coming from wt mice did not shed L-selectin (Figure 4).

The activation state of granulocytes found in the lower well after 16h of transendothelial migration in our assay was tested, and again, no sign of granulocyte activation was detected (data not shown). It could be that the time frame studied was not the right one. Most probably, endothelial cells may be a pre-requisite for granulocytes to be activated.

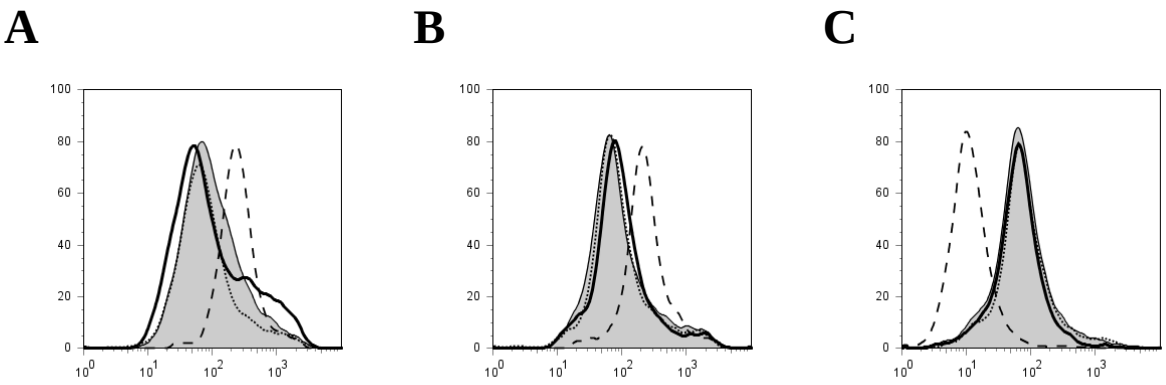


Figure 4: *Activation of granulocytes by tumor cells.* The figures **A** and **B** present Mac-1 expression at the surface of granulocytes coming from wt **(A)** or L-sel-/- mice **(B)** after 4h incubation at 37°C in the presence of tumor cells. The figure **C** presents MEL-14 staining of granulocytes coming from wt mice. Filled areas represent the staining of granulocytes incubated alone. Dotted lines represent granulocytes incubated in the presence of tumor cells at the ratio tumor cells : granulocytes = 1:1, while bold lines represent granulocytes after co-culture at the ratio 1:8. Dashed lines represent granulocytes after PMA activation.

Adhesion of tumor cells on endothelial cells in the presence of leukocytes

The adhesion of tumor cells to endothelium in the presence of leukocytes was tested. The establishment of new contacts between the tumor cell surface and endothelium is required for tumor cell invasion.

MC-38 cells adhere specifically on endothelial cells after 2-3 hours. Adhesion of MC-38 to endothelial cells (EC) was tested in the presence or absence of granulocytes for 3 h (Figure 5A). There was no apparent difference in the number of adherent tumor cells MC-38 on a confluent layer of EC irrespective of granulocytes -coming from either wt or Lsel -/- mice- (Figure 5B). The adhesion of tumor cells on endothelial cells was L-selectin independent. Moreover, we further showed that the granulocytes interacted with MC-38 cells in an L-selectin independent manner. This was confirmed by the analysis of leukocyte aggregates on tumor cells by cytospin (data not shown).

The promotion of tumor cell migration through endothelium in the presence of granulocytes (Figure 2 of Manuscript 1) is not correlated with an enhanced adhesion of tumor cells on endothelium. Another possibility is that the adhesion of tumor cells to endothelium in the presence of granulocytes may be flow and shear dependent, a well-known phenomenon for L-selectin molecules (Jadhav et al., 2001; Jadhav and Konstantopoulos, 2002). A further explanation could be that the adhesion of MC-38 to endothelium is not L- but P-selectin dependent.

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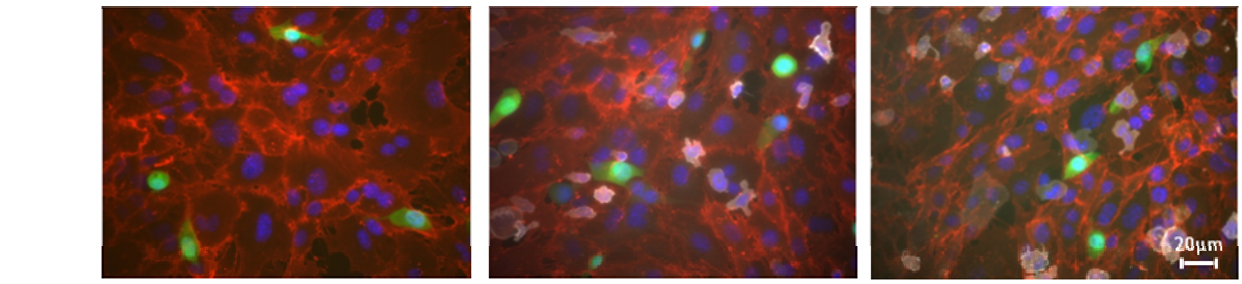

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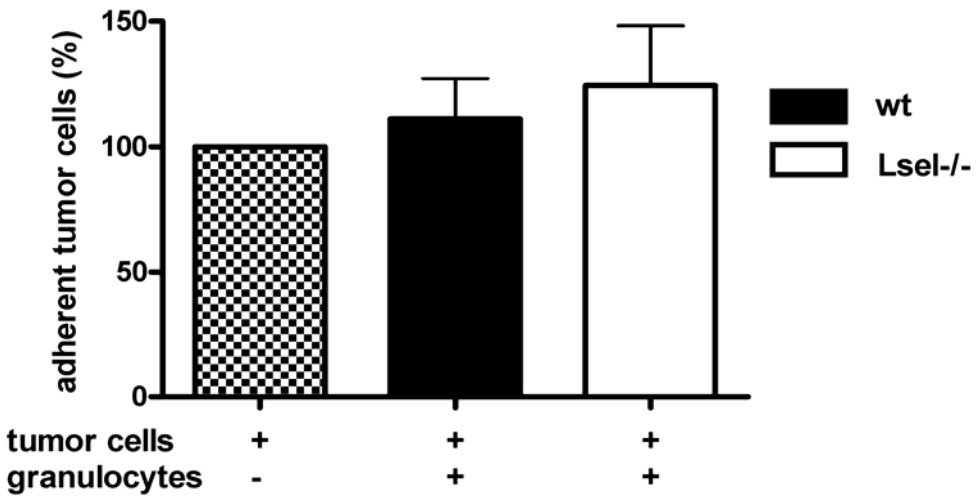

Figure 5: *Adhesion of tumor cells to endothelial cells in the presence of granulocytes.* **A** The pictures show the adhesion of MC-38GFP (green) on the EC layer stained with CD31 (red) in the presence / absence of granulocytes stained with Gr-1 (white). **B** The figure is a summary of two representative experiments (40x, 20 view fields). There was no statistical difference. *Columns*, mean; *bars*, SEM.

Migration of tumor cells through EC under a FCS gradient

We made the same transendothelial migration assay as described in the Manuscript 1 with the only difference that tumor cells were migrating towards a FCS gradient. The medium in the upper well was containing 0 % FCS whereas the medium in the lower well was containing 10 % FCS. Cells were mixed at the following ratios: for one part of tumor cells (20'000), ten parts of granulocytes (200'000) and five parts of monocytes (100'000) were added.

An enhancement of tumor cell migration in the presence of leukocytes was observed but in an L-selectin independent manner (Figure 6), whereas leukocytes transmigrate in an L-selectin dependent manner (Figure 7). Interestingly, tumor cells migrate in an L-selectin dependent manner only when TCM was present at the bottom well (see Manuscript 1). We suggest that the absence of L-selectin dependency in tumor cell transmigration is due to the absence of TCM. TCM is a conditioned medium, supernatant of the co-culture of endothelial cells and tumor cells. These findings suggest that interaction between endothelial cells and tumor cells leads to the release of factors that are primordial for tumor cell migration and that mimic the microenvironment. Thus, interaction of tumor cells with endothelial cells seems to be pivotal for the extravasation process. Moreover, L-selectin dependent leukocyte transmigration in this assay can be explained by an L-selectin local activation of the endothelial cells.

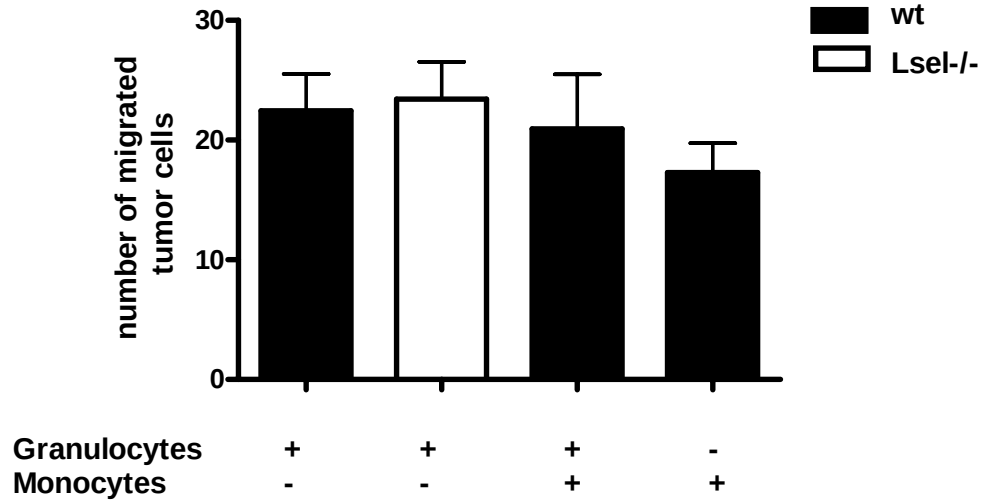

Figure 6: *Migration of tumor cells after 16h transmigration through EC in the presence of a FCS gradient.* This figure is a summary of at least six independent experiments. Migration of the tumor cells alone was normalized at 10 cells.

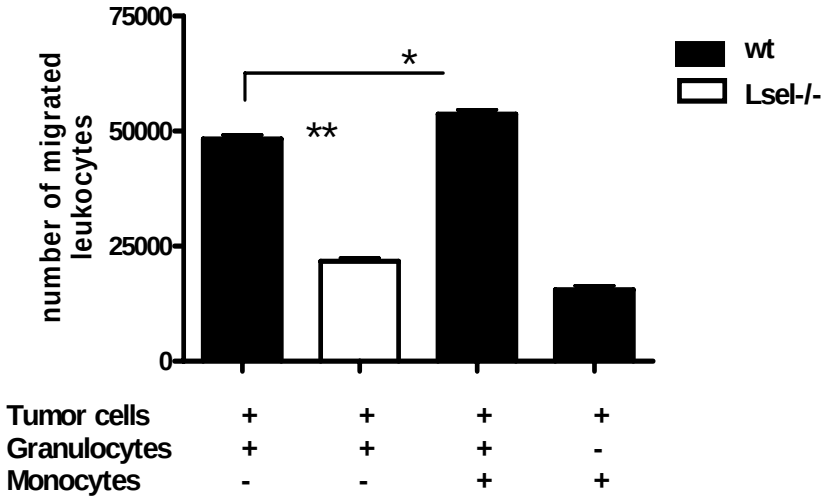

Figure 7: *Migration of leukocytes accompanying tumor cell transmigration in the presence of a FCS gradient.* This figure is a summary of at least three independent experiments. ** P<0.0001

Section III: Discussion

During carcinoma progression, tumor cells express altered glycosylation structures which are ligands for selectins. Metastasis is facilitated by the formation of a tumor cell embolus, consisting of L-selectin expressing leukocytes and P-selectin expressing platelets aggregated around tumor cells. Carcinomas carrying sLe^x structures can potentially bind L- and P-selectins. Platelets were shown to be active players in the metastatic spread and protect tumor cells from the clearance by innate immune cells (Borsig et al., 2001a; Garcia et al., 2007; Nieswandt et al., 1999). The attenuation of metastasis in mice deficient for L-selectin directly implicated leukocytes as facilitators of metastasis. However the nature and function of leukocytes in the tumor cell emboli remained to be elucidated.

Leukocytes were shown to be associated with tumor cells trapped in lungs (Läubli et al., 2006). In this work we have shown that the presence of innate immune cells (granulocytes, macrophages) around tumor cells was reduced in the lungs of mice deficient for L-selectin (see Manuscript 1). This finding indicates that cell-cell contacts between tumor cells and innate immune cells have a pivotal role for the contribution of metastasis. The association between tumor cell and innate immune cells has already been correlated with an enhancement of metastasis (Coussens et al., 2000; Queen et al., 2005). In a model of breast cancer, neutrophils were potential promoters of tumor progression *in vivo* (Queen et al., 2005). Neutrophil infiltration into tumor tissue led to the acquisition of a metastatic phenotype for benign murine fibrosarcoma cells (Tazawa et al., 2003). Depletion of tissue-associated macrophages by clodronate liposomes resulted in an inhibition of tumor growth (Zeisberger et al., 2006). Interestingly, the presence of bone marrow-derived cells modulated skin carcinogenesis (Coussens et al., 2000). An increase of metastasis was correlated with an enhanced infiltration of macrophages into the primary tumor (Lin et al., 2001). Macrophages recruited to the site of primary tumor are able to increase the metastatic potential of tumor cells for the colonization of distant sites. The presence of tumor-associated macrophages and neutrophils correlates with an increase of metastasis. Innate immune cells in the vicinity of tumor cells seem to be decisive for their further outcome. Little is known on the role of circulating monocytes associated with tumor cells. In our model, macrophages, granulocytes and monocytes associated with tumor cells may have a function important for the establishment of metastasis.

Granulocytes / monocytes promoted tumor cell transmigration through endothelium (see Manuscript 1), suggesting that leukocytes associated with tumor cells can increase migratory capacities of tumor cells in the blood vessel at the site of extravasation. Neutrophils were enhancing the *in vitro* invasive and *in vivo* metastatic potential of rat mammary

adenocarcinoma cells, by facilitating invasion into basement membrane (Welch et al., 1989). The observed facilitation of tumor cell transmigration through endothelium implicated L-selectin on leukocytes (see Manuscript 1). L-selectin interactions were pivotal for tumor cell migration through endothelium. In another study, L-selectin was transfected in carcinoma cells in which metastasis was infrequent and mostly localized in the liver (Qian et al., 2001). This L-selectin expression induced metastasis to lymph nodes where ligands for L-selectin, the adressins are expressed, showing the role of this cell-adhesion molecule in tumor spread. L-selectin ligands on the endothelium could be involved in MC-38 tumor cell extravasation. In order to further understand the underlying mechanism affecting tumor cell transmigration, it would be of interest to characterize the cells expressing L-selectin ligands and partners of leukocytes in tumor progression. Even though MC-38 and endothelial cells express L-selectin ligands (Läubli et al., 2006) it is still unknown whether tumor or endothelial cells interact with leukocytes for the observed facilitation of tumor cell extravasation. One possibility would be that leukocytes interact with the endothelium through L-selectin-PSGL-1 interactions and with tumor cells indirectly through binding with platelets. This would implicate a binding through endogenous ligands of endothelium. Studies using inhibition of selectins by single-dose heparin suggest that L-selectin on leukocytes may operate later as P-selectin on platelets during the metastatic cascade (Borsig et al., 2002). Leukocytes may be recruited by activated endothelium expressing new L-selectin ligands at the site where tumor cells are entrapped in the vasculature. Indeed L-selectin ligands can be induced on the endothelium during inflammation (Berg et al., 1998). Leukocytes have a high capability to go through endothelium during inflammation and L-selectin can modulate events downstream of leukocyte rolling and adhesion (Hickey et al., 2000). The signaling pathway induced by L-selectin on leukocytes involves an induced expression of TNF-α and IL-8 (Laudanna et al., 1994). This release of chemokines could modulate the microenvironment of the tumor emboli by increasing the survival of tumor cells and /or contribute to their subsequent extravasation into tissues.

In contrast to leukocytes that transmigrate through endothelial cells without disrupting them, many tumor cells induce endothelial cell retraction, and in some cases apoptosis or necrosis (Honn et al., 1994; Lee et al., 2003). It has been shown *in vitro* that the underlying endothelial cells retracted in contact with MC-38 tumor cells and leukocytes. These observations were successfully confirmed also *in vivo*. An enhancement of endothelial cell permeability was observed in presence of tumor cells and L-selectin on leukocytes. Human breast adenocarcinoma cells and Lewis lung cancer cells caused retraction of the endothelium (Honn

et al., 1994; Uchide et al., 2007). Vascular Endothelial Growth Factor (VEGF) produced by breast tumor cells induced endothelial cell retraction and redistribution of actin resulting in formation of stress fibers (Lee et al., 2003). Chemokine receptor CXCR4 and its ligand stromal cell derived factor 1 α (SDF-1α) were involved in this process (Lee et al., 2004). Tumor cells even induce apoptosis of endothelial cells and implicate a specific tumor-endothelial cell contact-dependent response (Heyder et al., 2002; Kebers et al., 1998). In our model, MC-38 in presence of leukocytes and endothelial cells could express chemokines or reactive oxygen species able to provoke endothelial cell retraction. Depletion of circulating monocytes led to an attenuation of metastasis (see Manuscript 1), meaning that circulatory monocytes are pivotal partners for the facilitation of tumor cell metastasis. L-selectin is implicated in monocyte activation by tumor cells (Putz and Mannel, 1996).This finding confirms that the observed tumor associated circulating monocytes have an important function *in vivo*. Taken together, recruited circulating monocytes may facilitate tumor progression by increasing the migratory capacities and /or survival of tumor cells and by acting on the permeability of the endothelial cells.

These findings open a way for new therapies of cancer metastasis. Leukocytes were recruited and have been shown to have a major role to promote metastasis. Therefore a treatment impairing the recruitment of inflammatory cells by tumor cells and their cell-cell contact could lead to reduction of metastasis. The limitation of such therapy is that it could affect the physiological role of leukocytes and platelets. It has been shown that a daily ingestion of aspirin reduces by half the risk of sporadic colon cancer (Dubois, 2000). Clinical studies report that inhibition of chronic inflammation by nonsteroidal anti-inflammatory drugs (NSAIDs), particularly COX-2 inhibitors could lead to an impairment of recruitment and function of inflammatory cells thereby an attenuation of metastasis (Fosslien, 2000; Pham-Nguyen et al., 1999). Finally, the targeting of the ligands of selectins could help to limit metastasis through blood vessels by inhibiting multiple interactions between tumor cells and selectin-bearing platelets, endothelium, and leukocytes. The injection of soluble form of E-selectin impaired lung metastasis in mice by interfering with the binding of colon carcinoma cells to endothelium (Mannori et al., 1997). Further, the blocking of sLe^x at the surface of tumor cells led to reduced metastatic properties of these cells (Fuster et al., 2003). Interestingly, heparin bound efficiently P- and L-selectin and inhibited their binding to sLe^x at the concentration used for anticoagulation (Koenig et al., 1998). Heparin treatment is often used as anticoagulant treatment for cancer patients suffering from associated thromboembolic complications (Smorenburg and Van Noorden, 2001) and beneficial effects of heparin or low-

molecular-weight heparin on attenuation of metastasis in humans were seen in clinical trials (Cosgrove et al., 2002). Studies in mouse models showed that the effect of heparin was not only based on its anticoagulant activity (Smorenburg and Van Noorden, 2001). A single dose of heparin led to a reduction of platelet-tumor cell aggregate formation in the lung vasculature that lasted for some hours and to a reduction of metastasis (Borsig et al., 2001a; Borsig et al., 2002). Use of low weight heparin in mouse models showed that heparin has an effect on attenuation of metastasis by targeting P- and L-selectin (Hostettler et al., 2007). Heparin should be injected when tumor cells are still in the vasculature for a therapy through its contribution to selectin inhibition. Heparin therapy through selectin inhibition and cell-cell interaction could be beneficial for cancer patients for treatment of metastatic progression.

Many evidences in mouse show that L-selectin on leukocytes promotes hematogenous metastasis. The contact between these immune cells with tumor cells and endothelial cells exerts a pivotal role for cancer progression. Inhibition of metastasis by impairment of immune cell recruitment by NSAIDs in human confirms the observations derived from the mouse model. Targeting the recruitment of inflammatory cells or the binding between tumor cells and inflammatory cells by inhibiting selectin interactions could prove to be a treatment for a metastatic disease.

Section IV: References

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Section V: Curriculum Vitae

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Date of birth: 10.07.1979
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EDUCATION

2003-07	PhD student at the University of Zürich since October 03
2002	Master : diploma of Biotechnology Engineer from the 'Ecole Supérieure de Technologie des Biomolécules de Bordeaux (ESTBB)', University Bordeaux 2, France
1997-99	Preparation for admission to engineering schools : 'Classes préparatoires'(Mathematics and Biology), Lycee J.Rostand, Strasbourg, France
1997	Scientific Baccalauréat with honours, Colmar, France

RESEARCH PROJECTS

At this date (4 years)	PhD at the University of Zürich • *Role of leukocytes during hematogenous metastasis*
2002 (1 year)	Work at the 'Université Libre de Bruxelles' (Brussels, Belgium) on a cancer research project • *Involvement of the S100A6 protein in glioma cell lines migration*
2001 (6 months)	Diploma at the Laboratory of Clinical and Experimental Immunology at Transgene S.A (Strasbourg, France) • *Project in Cancer Gene Therapy: 'Evaluation of the effect of genes expressed in tumor cells on the human dendritic cells' maturation'*
2000 (6 weeks)	Training in a R&D biochemistry laboratory at Novartis Crop Protection (Basel, Switzerland) • *Extraction, purification and preliminary biochemical characterizations of a target protein*

FOREIGN LANGUAGES

French	mother tongue
German	written and fluently spoken
English	written and fluently spoken

LEISURE ACTIVITIES AND PERSONAL INTERESTS

1996-2001	Coaching groups of young scouts and teenagers at many outdoor centres and children's holiday camps
Permanent	Music (cello player) and hiking